HPA-axis Functioning in Children:
A Psychobiological Perspective

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Chapter 1

General Introduction

A major player of the human stress system is the hypothalamic-pituitary-adrenal (HPA) axis, that produces cortisol as its primary hormonal end product (e.g., Lupien, McEwen, Gunnar, & Heim, 2009; Nicolson, 2007). Individual differences in cortisol production exist, both in adults and in children (e.g., de Weerth, Zijlmans, Mack, & Beijers, 2013a; Karlamangla, Friedman, Seeman, Stawksi, & Almeida, 2013; Kudielka, Hellhammer, & Wüst, 2009; van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012), and alterations in cortisol markers of HPA-axis functioning have been associated with mental and physical health (e.g., Bremmer et al., 2007; Burke, Davis, Otte, & Mohr, 2005; Buske-Kirschbaum et al., 2003; Hankin, Badanes, Abela, & Watamura, 2010; Jessop & Turner-Cobb, 2008; Luby et al., 2003; McEwen, 2008; Phillips, Ginty, & Hughes, 2013; Sephton & Spiegel, 2003). These links make it important to understand HPA-axis functioning, its dynamics, development, predictors, and correlates.

This is especially relevant in childhood for several reasons. First, HPA-axis functioning (as measured in cortisol) still seems to be developing during childhood and adolescence (e.g., Boyce & Ellis, 2005; Saridjan et al., 2010; Shirtcliff et al., 2012; Watamura, Donzella, Kertes, & Gunnar, 2004). Second, it can be modified by interventions (e.g., McLaughlin et al., 2015; Slopen, McLaughlin, & Shonkoff, 2014). Third, in childhood it is susceptible to environmental factors such as stress or adversity early in life, adverse caregiving, abuse and neglect, and sleeping and feeding arrangements (e.g., Beijers, Riksen-Walraven, & de Weerth, 2013a; Chaby, 2016; Fernald, Burke, & Gunnar, 2008; Hunter, Minnis, & Wilson, 2011; Loman & Gunnar, 2010; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2012; van der Vegt, van der Ende, Kirschbaum, Verhulst, & Tiemeier, 2009). Understanding HPA-axis functioning during childhood may hence offer entries for designing future prevention and intervention programs targeting children early in life.

There are still many gaps in knowledge regarding HPA-axis functioning in childhood. These gaps include unanswered questions about the dynamics and development of HPA-axis functioning, the associations between HPA-axis functioning and behavior, and the effect of environmental stress (early) in children's lives on the HPA-axis, on behavior, and on the associations between HPA-axis and behavioral functioning. The goal of this dissertation was to obtain a better understanding of these three topics in childhood. Specifically, it focuses on these topics in the first 6 years of life of typically developing children. A multidisciplinary psychobiological perspective, combining behavioral (observations and questionnaires), psychological (questionnaire), and biological (hormonal) measures was used to examine the aims of this dissertation.

In the remainder of this first chapter the following topics will be addressed. First, the theoretical background on HPA-axis functioning will be introduced (Section 1.1). Second, the three main and seven specific research aims of this dissertation will be presented,

with their theoretical background (Sections 1.2 to 1.4). This will be followed by an overview of the aims in Section 1.5. Third, the longitudinal research project through which the aims of this dissertation were pursued will be introduced (Section 1.6). This will be followed by an overview of the chapters of this dissertation (Section 1.7).

1.1 Hypothalamic-Pituitary-Adrenal (HPA) Axis Functioning

The HPA-axis consists of the hypothalamus and the pituitary gland in the brain, and the cortex of the adrenal glands in the torso. The hypothalamus produces the corticotrophin releasing hormone (CRH) which, in combination with the hormone arginine vasopressin (AVP), triggers the pituitary to secrete the adrenocorticotropic hormone (ACTH). This in turn stimulates the adrenal cortex to produce the hormone cortisol. Cortisol has an inhibiting function on the hippocampus, hypothalamus, and pituitary, and thus regulates HPA-axis activity by negative feedback loops (e.g., Lupien et al., 2009; Nicolson, 2007). Cortisol also has a regulatory function on many other processes, such as metabolism, memory, and the immune system (e.g., Sapolsky, Romero, & Munck, 2000). Cortisol can be measured reliably in a non-invasive, stress free manner by taking saliva samples. Saliva cortisol represents the free unbound cortisol concentration in plasma, that is, the biologically active fraction of the hormone cortisol (e.g., Kirschbaum & Hellhammer, 1994).

Cortisol is normatively secreted in a pulsatile fashion throughout the day. This production follows a well-defined 24 h cortisol circadian rhythm. This rhythm is characterized by high cortisol concentrations in the morning, including a sharp increase within 20 to 30 min after awaking, the cortisol awakening response (CAR; e.g., Fries, Dettenborn, & Kirschbaum, 2009). Thereafter cortisol concentrations gradually decline, most steeply in the first three hours after awakening, and reach the lowest point around midnight (e.g., Edwards, Clow, Evans, & Hucklebridge, 2001; Kirschbaum & Hellhammer, 1989).

In research, the diurnal cortisol rhythm is often operationalized by two markers (e.g., Saridjan et al., 2010; Watamura et al., 2004), total diurnal cortisol and the diurnal cortisol decline. Total diurnal cortisol represents the total amount of free cortisol to which the target tissue/physiology is exposed during the day. This marker is based on several diurnal samples and is sometimes called the total amount of cortisol or total cortisol concentration during the day. The diurnal cortisol decline represents the decline or slope of cortisol concentrations from morning to evening.

In addition to the cortisol circadian rhythm, the HPA-axis can also respond with augmented cortisol production to an acute stressful situation or “stressor”. By secreting cortisol, energy is mobilized to facilitate the individual’s response. When facing a stressful

situation the largest increase of saliva cortisol occurs from 21 to 30 min after stressor onset (Dickerson & Kemeny, 2004). Different types of stressors can result in an increase in cortisol concentrations: for example physical stressors (e.g., pain) as well as psychological stressors (e.g., al'Absi, Petersen, & Wittmers, 2002; Dickerson & Kemeny, 2004).

In laboratory settings psychological stressors that are most likely to result in increased cortisol concentrations in adults include motivational performance combined with social evaluation and uncontrollability (Dickerson & Kemeny, 2004). An example is motivated performance in cognitive or public speaking tasks in front of evaluating others (Dickerson & Kemeny, 2004). As such, the Trier Social Stress Task (TSST; Kirschbaum, Pirke, & Hellhammer, 1993), a public speaking/cognitive task combination, has been found to trigger a cortisol increase (Kirschbaum et al., 1993). A similar situation, motivational performance including forced failure in front of an evaluating judge, has recently been found to increase saliva cortisol concentrations in 5- to 6-year-old children (de Weerth et al., 2013a).

In research, the cortisol stress response can be divided in two components: the change in cortisol concentrations and the total cortisol production (Khoury et al., 2015). These components can be operationalized by two markers: by cortisol stress reactivity, that is, a representation of the increase in cortisol concentrations in response to the stressor, and by total stress cortisol, a representation of the total amount of cortisol that is secreted. This last marker is also called total stress cortisol and represents cortisol concentrations before, during, and after the acute stressor. Cortisol markers of the stress response are seen as indicators of physiological stress, and are not always associated with perceived distress (e.g., Dickerson & Kemeny, 2004).

Although both the cortisol circadian rhythm and cortisol stress responses are normative aspects of HPA-axis functioning in children and adults, individuals differ largely in both of them (e.g., de Weerth et al., 2013a; Karlamangla et al., 2013; Kudielka et al., 2009; van Hulle et al., 2012). To better understand HPA-axis functioning in children the current dissertation investigated these individual differences early in development. Specifically, it focused on unanswered questions regarding the dynamics and development of HPA-axis functioning, the associations between HPA-axis functioning and behavior, and the role of environmental stress (early) in children's lives on the HPA-axis, on behavior, and on the associations between HPA-axis and behavioral functioning.

1.2 Dynamics and Development

The first aim of this dissertation was to extend the current literature and address fundamental gaps in knowledge of the dynamics and development of HPA-axis functioning in

children. Specifically, the first aim of this dissertation was *to examine the dynamics and development of HPA-axis functioning in children up to/at the age of 6 (Aim 1).*

As indicated, the basal cortisol circadian rhythm and the cortisol stress response are both aspects of HPA-axis functioning (Section 1.1). For this reason associations between both are assumed. Research in adults has indeed shown associations between total diurnal cortisol concentrations and the cortisol stress response (Kidd, Carvalho, & Steptoe, 2014). But no support for associations between cortisol stress responses and diurnal cortisol decline or (basal) diurnal cortisol concentrations has been found (Kidd et al., 2014; van Eck, Nicolson, Berkhof, & Sulon, 1996). How both aspects of HPA-axis functioning are interrelated in children is largely unknown.

Earlier research with (primarily) adopted children between the age of 1.5 and 5 years found that blunted cortisol stress responses were associated with lower morning cortisol concentrations and blunted diurnal change (Koss, Mliner, Donzella, & Gunnar, 2016). But comparable research on the dynamics of HPA-axis functioning in typically developing children is scarce. Uncovering potential associations between the cortisol circadian rhythm and the cortisol stress response may make it possible to predict children's physiological capacity to respond with an efficient cortisol stress response in acute stressful situations. More specifically, basal HPA-axis functioning may inhibit or facilitate the ability to respond with an efficient cortisol stress response in an acute stressful situation.

Addressing this gap in the literature will further extend basic knowledge of HPA-axis functioning and will test the existing assumption that both patterns of cortisol production deriving from the same underlying mechanism, HPA-axis functioning, are associated. Hence, the current dissertation aims *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their cortisol circadian rhythm (Aim 1.1).*

In newborns, cortisol production does not yet follow the normative cortisol circadian rhythm. The newborn cortisol production follows a two-phase pattern with peaks unrelated to specific periods of the 24 h day-night sequence (Spangler, 1991). The normative cortisol circadian rhythm develops during the first year of life (e.g., Custodio et al., 2007; de Weerth & van Geert, 2002; de Weerth, Zijl, & Buitelaar, 2003; Ivars et al., 2015; Spangler, 1991) and cross-sectional studies suggest that the circadian rhythm continues to develop until at least the age of 3 (e.g., Saridjan et al., 2010; Watamura et al., 2004). Moreover, a longitudinal study in adolescence indicated that the cortisol circadian rhythm still shows developmental changes between the ages of 9 and 15 (Shirtcliff et al., 2012).

However, longitudinal studies on the development of the cortisol circadian rhythm after the first year of life are scarce. To complement the picture of development of the

cortisol circadian rhythm this development should be examined longitudinally in typically developing young children. By doing this it will also become possible to better understand the findings from cross-sectional child studies on diurnal HPA-axis functioning. This research may also be useful for diagnostic purposes and may facilitate future choices of research designs. Therefore, a goal of this dissertation was *to examine the longitudinal development of children's cortisol circadian rhythm from age 1 to 6 (Aim 1.2).*

1.3 Behavior

The second aim of this dissertation was *to examine the associations between HPA-axis functioning and behavior of children at the age of 6 (Aim 2).*

In stressful and threatening situations individuals differ widely in their behavioral and cortisol stress responses (de Veld, Riksen-Walraven, & de Weerth, 2014; de Weerth et al., 2013a; Kudielka et al., 2009; Wilson & MacLeod, 2003). For example, behaviorally, people can respond by adjusting their attention or by looking away from the stressor (de Veld et al., 2014; Wilson & MacLeod, 2003). In general, gazing has important functions, such as providing information, regulating interactions, expressing intimacy, exercising control, and facilitating goals (Kleinke, 1986). When confronted with a threatening situation, an individual's attentional vigilance can be affected (Wilson & MacLeod, 2003), including gazing behavior towards the threatening and stress inducing stimulus. Moreover, in a stressful situation gazing behavior may be associated with the cortisol stress response, and both stress responses may facilitate or inhibit one another.

Earlier research with university students has shown that larger increments of cortisol during a stressful interview were associated with more eye contact of the students with the interviewer (Sgoifo et al., 2003). However, studies of the association between cortisol stress responses and gazing behavior in stressful situations with children are scarce and have yielded contradictory findings. For example, in one study higher cortisol reactivity during a social challenge was associated with less looking at the interaction partner in 6- to 17-year-olds (Hessl, Glaser, Dyer-Friedman, & Reiss, 2006). In another study no support was found for a direct association between cortisol reactivity and gazing in 10-year-olds (de Veld et al., 2014).

Studies of associations between cortisol stress responses and gazing in young school-age children during a social evaluative, physiologically stressful situation will inform us of the role that gazing may play in these situations. Such research may contribute to understanding children's behavioral regulation strategies to deal with stressors. In the long run, this may offer an entry for training children in the use of behavioral strategies

to manage cortisol stress responses. Given these considerations, the first specific aim related to behavior was *to examine the association between 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 2.1).*

Alterations in cortisol markers of HPA-axis functioning have been associated with mental and physical health (e.g., Bremmer et al., 2007; Burke et al., 2005; Buske-Kirschbaum et al., 2003; Hankin et al., 2010; Jessop & Turner-Cobb, 2008; Luby et al., 2003; McEwen, 2008; Phillips et al., 2013; Sephton & Spiegel, 2003). However, it is less clear whether HPA-axis functioning in stressful situations is also associated with behavioral functioning in different contexts in typically developing young school-age children.

Earlier research suggested associations between children's physiological stress responses in acute stressful situations and their behavioral functioning in different contexts (e.g., Blair, Granger, & Razza, 2005; Conradt et al., 2014; Obradović, Bush, Stamperdahl, Adler, & Boyce, 2010; Spinrad et al., 2009). However, the results have been mixed and support for associations has not always been found (e.g., Alink et al., 2008; Spinrad et al., 2009). Examining associations between typically developing young school-age children's HPA-axis functioning in stressful situations and their behavioral functioning at school may help to further understand these associations. To address the link between cortisol stress responses and general behavior functioning the second specific aim related to behavior was *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 2.2).*

1.4 Environment

The third overall aim of this dissertation was to examine the role of environmental stress (early) in children's lives on HPA-axis functioning and behavior. Specifically, this aim was *to examine the role of environmental stress (early) in children's lives on behavior during a stressful situation and (the development of) HPA-axis functioning, as well as on the associations between HPA-axis and behavioral functioning up to/at the age of 6 (Aim 3).*

Both prenatally and postnatally, the child's environment is thought to influence its developing stress system (e.g., Loman & Gunnar, 2010; Seckl & Meaney, 2004). Environmental factors early in life may have long lasting effects on the developing offspring and are called programming effects (e.g., Lucas, 1991; Seckl & Meaney, 2004). During pregnancy, maternal distress, or more specifically, maternal (physiological) stress and anxiety, may affect the environment in utero (e.g., O'Donnell et al., 2012) resulting in a stressful environment for the fetus. This, in turn, may affect fetal development and the developing HPA-axis (e.g., Beijers, Buitelaar, & de Weerth, 2014).

Maternal distress may also play an important role during the early postnatal period when children are highly dependent on their caregivers. During this period, maternal distress may affect maternal behavior and caregiving and thereby create a stressful environment for the child. Loman and Gunnar (2010) suggested that adverse caregiving during this period may be a stressor for the child that, in turn, affects the developing stress neurobiology. Maternal distress may also lead to more experienced stress for the child if it results in a reduced ability of the mother to buffer her child from other stressful environmental influences.

Similarly, environmental stress early in children's lives, due to maternal prenatal and early postnatal distress, may also affect children's behavioral stress responses. In other words, prenatal and early postnatal environmental stress may program children's stress responses in general, including their behavioral stress responses. For example, children's level of attentional vigilance towards threatening stimuli (Wilson & MacLeod, 2003) or stressful situations, characterized by gazing behavior, may be affected by stress early in life. Suggestions for this idea may be found in research indicating that maternal prenatal stress was associated with gazing behavior of the infant during a peek-a-boo task (Lin, Crnic, Luecken, & Gonzales, 2014). Moreover, Loman and Gunnar (2010) suggested that caregiving early in children's postnatal lives may affect the development of children's rapid threat appraisal and response systems, as well as their developing behavior and emotion regulation systems. In other words, environmental stress in the early postnatal months of children's lives may not only affect their developing HPA-axis but also their threat appraisal and response systems and their behavioral and emotion regulation systems, possibly including their behavioral stress responses, such as their gazing behavior in stressful situations.

Previous research indeed found associations between maternal distress during pregnancy and offspring's circadian cortisol concentrations (e.g., Gutteling, de Weerth, Buitelaar, 2005; O'Donnell et al., 2013; van den Bergh, van Calster, Smits, van Huffel, & Lagae, 2008). Associations were also found between early postnatal maternal parenting stress and anxiety disorders, and children's total diurnal and pre-stressor cortisol concentrations, respectively (e.g., Saridjan et al., 2010; Warren et al., 2003). Associations between maternal prenatal distress and child cortisol stress responses also have been found (e.g., Davis, Glynn, Waffarn, & Sandman, 2011; Gutteling, de Weerth, & Buitelaar, 2004; Leung et al., 2010; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011). Additionally, and in line with the above, mothers with an anxiety disorder were less sensitive and more intrusive than healthy controls, and had children with higher cortisol stress responses (Feldman et al., 2009).

However, several questions remain. First, not much is known about the associations between maternal prenatal and early postnatal distress and the development of the cor-

tisol circadian rhythm during the first 6 years of the child's life. Second, findings regarding the associations between prenatal maternal distress and child cortisol stress responses provide a mixed picture, and research on the associations between early postnatal maternal distress and children's cortisol stress responses is scarce. Also, research on the associations between maternal distress (prenatal and early postnatal stress and anxiety) and young school-age children's behavioral stress response in the form of gazing seems lacking.

Investigating these associations may provide further support for early programming hypotheses (e.g., Lucas, 1991; Seckl & Meaney, 2004) and the idea of stress early in life as a predictor of later HPA-axis functioning (e.g., Chaby, 2016; Loman & Gunnar, 2010). Moreover, studying these associations in a typically developing middle class sample will increase our understanding of the associations between prenatal and early postnatal stress and behavioral stress responses as well as (the development of) HPA-axis functioning.

To address these aspects this dissertation focused on the following two aims: *examine the associations between maternal prenatal and early postnatal distress and (the longitudinal development of) children's cortisol circadian rhythm from age 1 to 6 (Aim 3.1); and examine the associations between maternal prenatal and early postnatal distress and 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 3.2).*

A third aim regarding the role of the environment was *to examine the moderating role of current maternal parenting stress on the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 3.3).* Although, as described, environmental stress early in children's lives may be linked with their behavior in a stressful situation and their developing HPA-axis, stress in the current environment of young school-age children may also play a relevant role.

As indicated in Section 1.3, studies of the association between children's cortisol stress responses in acute stressful situations and their behavioral functioning in a different context have produced mixed results (e.g., Alink et al., 2008; Blair et al., 2005; Conradt et al., 2014; Obradović et al., 2010; Spinrad et al., 2009). This may be explained by a moderating role of the current home environment. Maternal distress may affect the stressfulness of the child's current home environment by shaping maternal behavior. Indeed, parenting stress has been found to be associated with stricter discipline, less nurturing, more laxness, and lower parental expectations of the child as indicated by the parent (Anthony et al., 2005; Guajardo, Snyder, & Petersen, 2009). Stress in the current home environment may, in turn, affect the associations between children's HPA-axis and behavioral functioning. Investigating the moderating role of environmental stress at home in the association between cortisol stress responses and behavioral functioning in young school-age children may shed light on the earlier mixed findings for this association. It will also strengthen our knowledge of factors that facilitate or inhibit this association.

1.5 Study Aims

This dissertation addressed the following three previously described overarching, and seven specific research aims:

1. To examine the **dynamics and development** of HPA-axis functioning in children up to/at the age of 6.
    1.1 To examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their cortisol circadian rhythm (Chapter 2).
    1.2 To examine the longitudinal development of children's cortisol circadian rhythm from age 1 to 6 (Chapter 3).
2. To examine the associations between HPA-axis functioning and **behavior** of children at the age of 6.
    2.1 To examine the association between 6-year-old children's cortisol stress responses and gazing during an acute stressor (Chapter 4).
    2.2 To examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Chapter 5).
3. To examine the role of **environment**al stress (early) in children's lives on behavior during a stressful situation and (the development of) HPA-axis functioning, as well as on the associations between HPA-axis and behavioral functioning up to/at the age of 6.
    3.1 To examine the associations between maternal prenatal and early postnatal distress and (the longitudinal development of) children's cortisol circadian rhythm from age 1 to 6 (Chapter 3).
    3.2 To examine the associations between maternal prenatal and early postnatal distress and 6-year-old children's cortisol stress responses and gazing during an acute stressor (Chapter 4).
    3.3 To examine the moderating role of current maternal parenting stress on the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Chapter 5).

1.6 The Longitudinal BIBO Project

All research aims of this dissertation were pursued in the BIBO project (Radboud University). BIBO stands for the Dutch translation of Basal Influences on Infant Development (in Dutch: Basale Invloeden op de Baby Ontwikkeling). BIBO is an ongoing prospective longitudinal project following mother-child dyads from pregnancy on. The primary focus of the BIBO project is to study the effects of the (early life) environment on children's psychobiological development. Inclusion criteria at the start of the study were a clear understanding of Dutch, no drug use during pregnancy, no current health problems (physical or mental), an uncomplicated singleton pregnancy with a term delivery, and an infant 5-minute Apgar score of 7 or higher (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2010). In this way, a cohort of typically developing children from middle class families was recruited. At the start of the project 220 healthy born children and their mothers were enrolled and 193 dyads were still in the project 3 months postpartum (for details, see Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011a, 2011b; Beijers et al., 2013a; Beijers, Riksen-Walraven, Putnam, de Jong, & de Weerth, 2013b). At child age 6, 149 children participated in a school visit including a social evaluative stress test. Reasons for non-participation are described in Chapters 2, 4, and 5.

In the current dissertation data from the prenatal period and the first 6 postnatal years were used. Figure 1.1 gives an overview of the behavioral, psychological, and biological measures that were used per developmental period. Prenatal maternal distress was measured with maternal self-reports of general and pregnancy-related stress and anxiety and with the maternal diurnal cortisol rhythm derived from saliva samples. These measures were collected around the 37th week of pregnancy. Early postnatal maternal distress was measured with maternal self-reports of stress and anxiety when the child was 3 and 6 months old. At child age 1 these maternal questionnaires were repeated and at age 2.5 maternal distress was measured with a self-report of maternal anxiety. At these two ages (child age 1 and 2.5 years) mothers were also asked to collect child saliva samples to determine the cortisol circadian rhythm. When the child was 6 years old maternal distress was again determined with a maternal self-report of anxiety. Maternal feelings of parenting stress were also determined with self-report. The child's cortisol circadian rhythm was assessed again, and teacher reports of child behavioral functioning (internalizing, externalizing, and prosocial behavior) were collected. Moreover, children participated in the Children's Reactions to Evaluation Stress Test (CREST; de Weerth et al., 2013a) during a school visit. Cortisol stress responses were assessed by collecting saliva; the behavioral stress response in the form of gazing was scored from videotapes of the task. Figure 1.1 gives an overview of the measures for each developmental period.

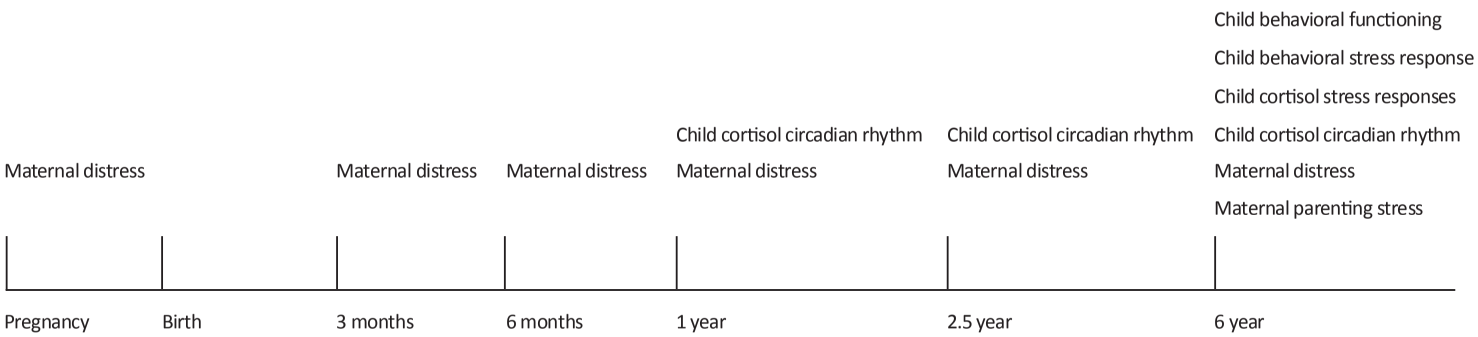

Figure 1.1 | Measures used in the dissertation per developmental period

1.7 Thesis Outline

The research aims of this dissertation are addressed in four empirical chapters (Chapters 2 to 5). Table 1.1 gives an overview of the dissertation aims per empirical chapter.

Chapter 2: Associations between circadian and stress response cortisol in children.
The goal of this chapter was *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their cortisol circadian rhythm (Aim 1.1)*. To address this goal data on the 6-year-old children's cortisol stress responses during the social evaluative stress test (CREST, de Weerth et al., 2013a) as well as on their cortisol circadian rhythm were used.

Chapter 3: Development of the cortisol circadian rhythm in the light of stress early in life.
The first goal of this chapter was *to examine the longitudinal development of children's cortisol circadian rhythm from age 1 to 6 (Aim 1.2).* The second goal was *to examine the associations between maternal prenatal and early postnatal distress and (the longitudinal development of) children's cortisol circadian rhythm from age 1 to 6 (Aim 3.1).* To address these goals, data on the children's cortisol circadian rhythm at 1 year, 2.5 years, and 6 years of age was used. Moreover, data on maternal prenatal and early postnatal distress was used.

Chapter 4: Child stress responses at age 6 in the light of stress early in life.
The first goal of this chapter was *to examine the associations between maternal prenatal and early postnatal distress and 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 3.2).* The second goal was *to examine the association between 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 2.1)*. To address these goals, data on the 6-year-old children's cortisol stress responses and their gazing behavior during the social evaluative stress test were used. Moreover, data on maternal prenatal and early postnatal distress was used.

Chapter 5: Cortisol stress responses and children's behavioral functioning at school.
The first goal of this chapter was *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 2.2).* The second goal was *to examine the moderating role of current maternal parenting stress on the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 3.3).* To address these goals, data on the 6-year-old children's cortisol stress responses during

the social evaluative stress test and teacher reports on their behavioral functioning (internalizing, externalizing, and prosocial behavior) were used. Moreover, data on maternal feelings of parenting stress at child age 6 were used.

Chapter 6: General discussion.
Following the four empirical chapters, Chapter 6 will provide a general discussion. In this chapter insights in the three main research aims will be discussed. Moreover, early childhood as a developmental period for HPA-axis functioning, possibilities to translate the obtained knowledge into clinical practice, strengths and limitations, suggestions for future research, and conclusions will be addressed.

Table 1.1 | *Overview of the Dissertation Aims per Empirical Chapter*

Chapter	Aim 1	Aim 2	Aim 3	Topic
	Dynamics and Development	*Behavior*	*Environment*	
2	Aim 1.1			Associations between circadian and stress response cortisol
3	Aim 1.2		Aim 3.1	Development of the cortisol circadian rhythm in the light of stress early in life
4		Aim 2.1	Aim 3.2	Cortisol and behavioral stress responses in the light of stress early in life
5		Aim 2.2	Aim 3.3	Cortisol stress responses and behavioral functioning at school; the role of current stress

Chapter 2

Associations Between Circadian and Stress Response Cortisol in Children

Based on:

Simons, S. S. H., Cillessen, A. H. N., & de Weerth, C. (2017). Associations between circadian and stress response cortisol in children. *Stress, 20*(1), 69-75. doi:10.1080/10253890.2016.1276165

Abstract

Hypothalamic-pituitary-adrenal (HPA) axis functioning is characterized by the baseline production of cortisol following a circadian rhythm, as well as by the superimposed production of cortisol in response to a stressor. However, it is relatively unknown whether the basal cortisol circadian rhythm is associated with the cortisol stress response in children. Since alterations in cortisol stress responses have been associated with mental and physical health, this study investigated whether the cortisol circadian rhythm is associated with cortisol stress responses in 6-year-old children. To this end, 149 normally developing children (M_{age} = 6.09 years; 70 girls) participated in an innovative social evaluative stress test that effectively provoked increases in cortisol. To determine the cortisol stress response, six cortisol saliva samples were collected and two cortisol stress response indices were calculated: total stress cortisol and cortisol stress reactivity. To determine children's cortisol circadian rhythm eight cortisol circadian samples were collected during two days. Total diurnal cortisol and diurnal cortisol decline scores were calculated as indices of the cortisol circadian rhythm. Hierarchical regression analyses indicated that higher total diurnal cortisol as well as a smaller diurnal cortisol decline, were both uniquely associated with higher total stress cortisol. No associations were found between the cortisol circadian rhythm indices and cortisol stress reactivity. Possible explanations for the patterns found are links with children's self-regulatory capacities and parenting quality.

Keywords: cortisol circadian rhythm, cortisol stress responses, HPA-axis functioning, 6-year-olds, diurnal cortisol, stress cortisol

2.1 Introduction

The hypothalamic-pituitary-adrenal (HPA) axis has as primary hormonal end product, the hormone cortisol (e.g., Lupien, McEwen, Gunnar, & Heim, 2009). Baseline HPA-axis functioning is characterized by a 24 h cortisol circadian rhythm consisting of high morning cortisol concentrations followed by a gradual decline till nadir (e.g., Edwards, Clow, Evans, & Hucklebridge, 2001; Kirschbaum & Hellhammer, 1989). Superimposed on this rhythm, the HPA-axis also produces cortisol in response to stressors (e.g., Dickerson & Kemeny, 2004; Nicolson, 2007). Both of these normative patterns are characterized by individual differences (e.g., Karlamangla, Friedman, Seeman, Stawksi, & Almeida, 2013; Kudielka, Hellhammer, & Wüst, 2009). This study investigated associations between these two aspects of HPA-axis functioning in normally developing children at the beginning of elementary school.

In many countries the beginning of elementary school marks the start of achievement monitoring, raising teachers' and parents' expectations. Furthermore, at this age impression management/self-presentation is used (first seen in 5-year-olds; Engelmann, Herrmann, & Tomasello, 2012) and feelings of relief and regret develop (between age 4 and 7; Weisberg & Beck, 2012). Hence, assumingly at this age children become more exposed and sensitive to social evaluative stressors that can trigger a cortisol stress response (Dickerson & Kemeny, 2004).

Since alterations in cortisol stress responses have been associated with mental and physical health (e.g., Buske-Kirschbaum et al., 2003; Hankin, Badanes, Abela, & Watamura, 2010; Luby et al., 2003) it is important to understand its correlates. Uncovering associations between the cortisol circadian rhythm and cortisol stress responses may reveal how certain aspects of circadian cortisol dynamics may in part be predictive of children's physiological capacity to cope with stressors. Dynamics of the cortisol circadian rhythm may facilitate or inhibit efficient cortisol stress responses.

Research in adults between the age of 54 and 76, showed positive associations between total daily cortisol concentrations and the magnitude of the cortisol stress response. However, no associations were found between diurnal cortisol decline and the magnitude of the cortisol stress response (Kidd, Carvalho, & Steptoe, 2014) or, in 27- to 57-year-olds, between (basal) diurnal cortisol concentrations and the cortisol stress response (van Eck, Nicolson, Berkhof, & Sulon, 1996).

Research in 1.5- to 5-year-old (predominantly adopted) children showed that both lower morning cortisol concentrations and blunted diurnal change were associated with blunted cortisol stress responses (Koss, Mliner, Donzella, & Gunnar, 2016). However, re-

search on the dynamics of HPA-axis functioning in normally developing children at the beginning of elementary school is still scarce.

Hence, we investigated associations between the cortisol circadian rhythm and cortisol stress responses in normally developing 6-year-olds. An age-appropriate social evaluative stressor (de Weerth, Zijlmans, Mack, & Beijers, 2013a), was used to provoke cortisol stress responses. The cortisol circadian rhythm was operationalized as total diurnal cortisol and diurnal cortisol decline (e.g., Nater, Hoppmann, & Scott, 2013; Saridjan et al., 2010; Simons, Beijers, Cillessen, & de Weerth, 2015; Watamura, Donzella, Kertes, & Gunnar, 2004). Associations of these two circadian indices with two indices of cortisol stress responses (total stress cortisol and cortisol stress reactivity) were investigated. Directions of these associations, as well as the interactive effect of both circadian indices, were explored.

2.2 Methods

2.2.1 Participants

This study was part of an ongoing longitudinal project focusing on psychobiological factors in child development (BIBO project; Radboud University). The original project and the 6-year data collection were approved by the Institutional Review Board, which adheres to the Helsinki Declaration (ECG 300107 and ECG 22111/130112, respectively). Originally, a total of 220 pregnant mothers enrolled in the project, and 193 mother-child dyads were still participating when the child was 3 months old (for details, see Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011a; Beijers, Riksen-Walraven, & de Weerth, 2013a; Beijers, Riksen-Walraven, Putnam, de Jong, & de Weerth, 2013b). Around the 6th birthday of the child, the 188 mother-child dyads still in the project were invited to take part in the current data collection. Parents who accepted the invitation were asked to sign an informed consent form. Of the invited group, 149 children participated in a school visit containing a social evaluative stress test (M_{age} = 6.09; *SD* = 0.14; *Min* = 5.87, *Max* = 6.85; 70 girls). Reasons for non-participation were: the school or the child chose not to participate (*n* = 4), the family had moved abroad (*n* = 3), or other reasons (e.g., parents perceived the procedure as too challenging for their child, parents perceived the study as too intensive, or personal reasons, *n* = 32). The group of children that did not participate in the current data collection after the invitation (*n* = 39) did not differ significantly from the participating group in maternal educational level during pregnancy, maternal age at delivery, gender of the child, and child age 4 temperament (Children's Behavior Questionnaire short form; CBQ short form; Putnam & Rothbart, 2006), all *p's* > .050.

Of the 149 children, five were excluded from the current study because of irregularities in the six cortisol saliva samples collected during the school visit to determine the cortisol stress response. Specific reasons were: the use of medication that potentially affects cortisol concentrations (n = 3), large time deviations from the saliva sampling protocol (n = 1), and all six samples were missing because the child refused to participate in saliva sampling (n = 1). This yielded a final sample of 144 children (M_{age} = 6.09; SD = 0.14; Min = 5.87, Max = 6.85; 68 girls).

2.2.2 Procedure

2.2.2.1 Cortisol circadian rhythm

To determine children's cortisol circadian rhythm, mothers were asked to collect eight saliva samples of their child during two weekend days at four predefined time points: immediately after the child woke up (T1), at 11:00 h (T2), at 15:00 h (T3), and at 19:00 h (T4) (Simons et al., 2015). Of the 144 mother-child dyads in the final sample, 138 participated in collecting circadian saliva samples.

2.2.2.2 Cortisol stress responses

To assess cortisol stress responses children were exposed to a social evaluative stress paradigm, the Children's Reactions to Evaluation Stress Test (CREST; de Weerth et al., 2013a; Simons, Cillessen, & de Weerth, 2017a). The CREST contains elements of unpredictability and uncontrollability, and is carried out in front of a judge that evaluates the child's performance (social-evaluation). The test consists of three forced-failure tasks with a total duration of 15 min, followed by a 5-minute period in which the child is anticipating a final performance evaluation by the judge. Specifically, the child is asked to perform three tasks in front of a judge who evaluates the child's performance and rewards good performance with a present chosen in advance by the child. In the first task, the child is asked to stand as still as possible with an alarm clicked onto his/her clothing. The child is told that the alarm will go off with movement. However, independent of actual movement the alarm goes off twice within the total task duration of 1 min. In the second task, the child is played a tape of a story in which eight animals are mentioned, each followed by a pause. In this task (3 min), the child is asked to provide the sound made by each animal in the subsequent pause, and is told that the judge will show a green card upon good performance. However, independently of the child's actual performance the judge only shows the green card in three out of eight sounds. In the third task (3 min) the child is asked to build a pyramidal tower of horizontally lying cans, imitating a tower shown by the researcher. The child is told that this task is considered easy by peers, whereas in reality it is almost impossible. After these three tasks the judge leaves for 5 min to decide on

the child's performance. Upon return, the judge rewards the child for good performance with the chosen present (for more details, see de Weerth et al., 2013a). Subsequentely, children are debriefed and assured again that they performed well. This procedure was found to be stressful in an earlier independent study on 5- to 6-year-old children (*n* = 42), as indicated by a significant increase in cortisol concentrations in response to the test (de Weerth et al., 2013a). Following the test, children were allowed to draw and watch movies during a 25-minute recovery phase, followed by 25 min in which they participated in tasks unrelated to the present study. This procedure took place in the afternoon (start of visits between 12:15 and 15:15 h; *M* = 13:34 h, *SD* = 0:21) of a regular school day in a mobile laboratory parked near the child's school (or home, *n* = 7 of 144).

During this procedure, six saliva samples (C1-C6) were collected from each child. C1 was collected just before the CREST started. C2-C6 were collected 15, 25, 35, 50 and 58 min after the start of the CREST, respectively. As in the original CREST paradigm, C1 and C2 represent baseline cortisol concentrations (de Weerth et al., 2013a). Children were asked to refrain from eating, drinking, or being physically active in the 30 min prior to the school visit. Cortisol increases in saliva as a response to a stressor can best be measured from 21-30 min after stressor onset on (Dickerson & Kemeny, 2004). Hence, C3 and C4 represent cortisol concentrations in response to the stressor. C5 and C6 represent recovery cortisol concentrations. To control for potential effects of illnesses on cortisol concentrations, the procedure was rescheduled if a child was ill. Children with a cold on the testing day (*n* = 7 of 144) did not differ significantly in their cortisol concentrations from the rest of the group (all *p's* > .050).

2.2.3 Measures

2.2.3.1 Cortisol analyses

Cortisol saliva samples for both the cortisol circadian rhythm and cortisol stress responses were collected using eye sponges (BD Visispeare, Waltham, MA; de Weerth, Jansen, Vos, Maitimu, & Lentjes, 2007). At each saliva sampling moment, participants were asked to put two eye sponges in their mouth and saturate them with saliva. Back at the lab, eye sponges were centrifuged to obtain the saliva which was stored in a freezer (-25 °C). Cortisol analyses were carried out at the Laboratory of Endocrinology of the University Medical Center Utrecht (for details, see Simons et al., 2015; 2017a).

2.2.3.1.1 Cortisol circadian rhythm

Of the potential 1104 circadian saliva samples of the 138 children, 1023 analyzable samples were obtained. Of the analyzable samples 69 were eliminated (6.7%) because of large deviations from standard sampling times (for details, see Simons et al., 2015),

illnesses, the use of medication that potentially affects cortisol concentrations, or biologically extreme concentrations. Figure 2.1 presents the average diurnal cortisol concentrations (nmol/L) as well as standard errors for each of the four diurnal cortisol saliva sampling moments.

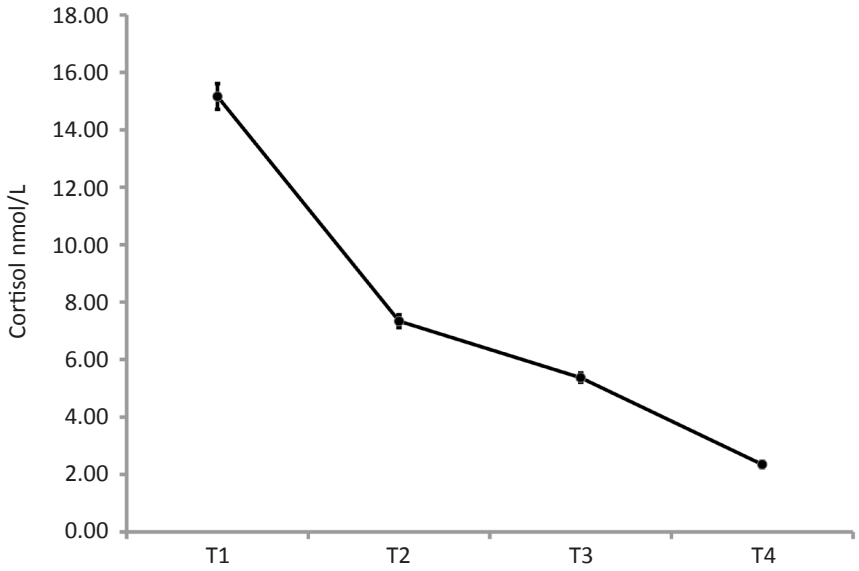

Figure 2.1 | Diurnal cortisol concentrations (nmol/L) for each of the four sampling moments (based on average scores over both sampling days). Error bars stand for one standard error above and one beneath the mean of each of the four diurnal sampling moments.

Two often-used indices of the cortisol circadian rhythm were calculated: total diurnal cortisol and diurnal cortisol decline (e.g., Nater et al., 2013; Saridjan et al., 2010; Simons et al., 2015; Watamura et al., 2004). Total diurnal cortisol was calculated as the area under the curve as follows: $AUC_{diurnal}$ = ((T2 + T1) x time between sample T1 and T2/2 + (T3 + T2) x time between sample T2 and T3/2 + (T4 + T3) x time between sample T3 and T4/2). Diurnal cortisol decline was calculated as sample T1 minus T4. These measures were averaged across days (Simons et al., 2015; Watamura et al., 2004). Spearman's *rho* correlations across days were *rho* = .62, $p < .001$, and *rho* = .29, $p = .004$, for total diurnal cortisol and diurnal cortisol decline, respectively. These correlations are in line with previous research in childhood and adolescence describing that total diurnal cortisol concentrations (AUC measures) are moderately stable across two days whereas the stability of diurnal decline scores is lower (Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012).

In addition, since both indices of the cortisol circadian rhythm were significantly correlated with the length of the day (time distances between samples T1 and T4 in minutes; Spearman's *rho* = .23, *p* = .016 and Spearman's *rho* = .27, *p* = .002, for total diurnal cortisol and diurnal cortisol decline, respectively), both total diurnal cortisol and diurnal cortisol decline were also calculated corrected for the length of the day. This was done by saving the standardized residuals of regression analyses predicting the cortisol circadian measures based on the length of the day (based on de Veld, Riksen-Walraven, & de Weerth, 2012; Schuetze, Lopez, Granger, & Eiden, 2008; Simons et al., 2017a). Using these corrected indices of the cortisol circadian rhythm in the main regression analyses resulted in comparable results to those with uncorrected indices. To facilitate interpretation of the results, findings of analyses using the uncorrected indices will be reported in the results section.

2.2.3.1.2 Cortisol stress responses

Of the potential 864 cortisol stress response saliva samples of the 144 children, 843 analyzable samples were collected. In Figure 2.2, mean cortisol concentrations (nmol/L) and standard errors for each of the six cortisol sampling moments can be found. A paired samples *t*-test indicated that the CREST induced a significant increase in cortisol concentrations from baseline (lowest of C1 and C2; *M* = 6.06, *SD* = 2.70) to peak response concentrations (highest of C3 and C4; *M* = 7.12, *SD* = 3.79), *t*(141) = -4.41, *p* < .001, Cohen's *d* = 0.37.

Two indices of the cortisol stress response were calculated: total stress cortisol and cortisol stress reactivity. Total stress cortisol was calculated as the area under the curve over the six saliva samples as follows: AUC_{stress} = ((C2 + C1) x 15/2 + (C3 + C2) x 10/2 + (C4 + C3) x 10/2 + (C5 + C4) x 15/2 + (C6 + C5) x 8/2). Cortisol stress reactivity was calculated as the standardized residuals of a regression predicting peak response cortisol from the baseline (cf., de Veld et al., 2012; Schuetze et al., 2008; Simons et al., 2017a). The correlation between baseline and peak response cortisol concentrations was Spearman's *rho* = .69, *p* < .001. Both indices of the cortisol stress response (total stress cortisol and cortisol stress reactivity) were log transformed before they were used in the analyses.

2.2.3.2 Confounders

Child gender (i.e., biological sex) and maternal educational level at child age 6 were determined as potential confounders. Child gender was scored as 0 (*girl*) or 1 (*boy*), and maternal educational level was scored as the mother's highest educational level from 1 *(primary)* to 8 *(university).*

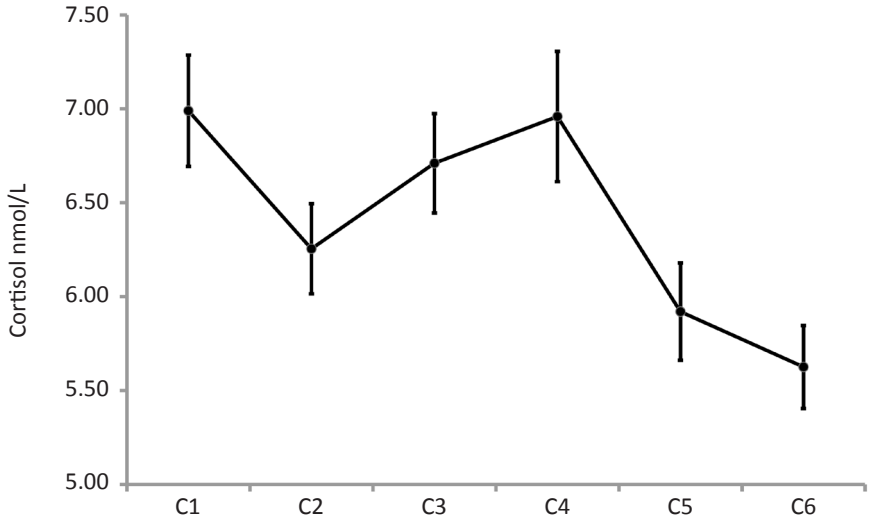

Figure 2.2 | Stress response cortisol concentrations (nmol/L) per sampling moment. Error bars stand for one standard error above and beneath the mean of each of the six stress response sampling moments. Note that this is an adapted figure from Simons et al. (2017a). Data are reprinted with permission of the publisher: John Wiley and Sons. License number: 4002560272811.

2.3 Results

2.3.1 Preliminary Analyses

Children who participated in the CREST in the mobile lab parked at their home (n = 7 of 144) did not differ significantly on the outcome or predictor variables from children that participated with the lab parked at their school (all p's > .050) and were therefore included in the analyses. Table 2.1 presents descriptive statistics of the untransformed study variables.

In Table 2.2, Spearman correlations between all study variables are presented. Total diurnal cortisol ($AUC_{diurnal}$) was positively associated with total stress cortisol (AUC_{stress}; Spearman's *rho* = .24, p = .013). Higher total diurnal cortisol concentrations were associated with higher total stress cortisol concentrations. Potential confounders (child gender and maternal educational level) were not significantly correlated with an outcome variable (all p's > .050; total stress cortisol or cortisol stress reactivity) and were hence left out of the main regression analyses (Tabachnick & Fidell, 2007). Note that main regression analyses including child gender (as confounder or predictor variable) resulted in similar findings to those reported in the main analyses section.

Table 2.1 | *Descriptive Statistics of all Study Variables*

	N	*M*	*SD*	*Min*	*Max*
Confounders					
Child gender (% girls)	144	47.2%			
Maternal educational level[a]	140	6.78	1.39	3.00	8.00
Predictors					
Total diurnal cortisol (nmol/L)	111	4837.33	1371.71	2518.25	10801.13
Diurnal cortisol decline (nmol/L)	126	12.98	4.96	-0.35	26.00
Outcomes					
Total stress cortisol (nmol/L)	134	375.80	169.48	73.80	1474.50
Cortisol stress reactivity[b] (nmol/L)	142	0.00	1.00	-1.77	5.15

Note. [a]scored from 1 = *primary* to 8 = *university*.
[b]*M* = 0 and *SD* = 1 because these are standardized residuals.

Table 2.2 | *Spearman Correlations Between all Study Variables*

	1.	2.	3.	4.	5[a].
Confounders					
1. Child gender					
2. Maternal educational level	-.00				
Predictors					
3. Total diurnal cortisol	-.08	.12			
4. Diurnal cortisol decline	-.02	.13	.68***		
Outcomes					
5. Total stress cortisol[a]	-.04	-.05	.24*	.14	
6. Cortisol stress reactivity[a]	.10	-.12	-.16^{+}	-.08	.46***

Note. [a]log transformed.
$^{+}$ $p < .100$, * $p < .050$, *** $p < .001$.

Of the 144 children, 33 dropped out of both main regression analyses because of missing predictor (diurnal decline $n = 18$, $AUC_{diurnal}$ $n = 33$) and outcome variables (AUC_{stress} $n = 10$, cortisol stress reactivity $n = 2$). These 33 children did not differ significantly from the 111 children remaining in the main analyses on maternal educational level, maternal age, child gender, or age 4 temperament (CBQ short form; Putnam & Rothbart, 2006), all *p's* > .050.

2.3.2 Main Analyses

Two hierarchical linear regression analyses were conducted. In the first analysis, total stress cortisol (AUC_{stress}) was predicted from the two indices of the cortisol circadian rhythm (AUC-

$_{diurnal}$ and diurnal cortisol decline) in Step 1, and the interaction of the two indices of the cortisol circadian rhythm in Step 2. The model of Step 1 was significant, $F(2, 102) = 13.83$, $p < .001$, $R^2 = .21$. Both total diurnal cortisol ($\beta = .59$, $p < .001$) and diurnal cortisol decline ($\beta = -.25$, $p = .030$) significantly predicted total stress cortisol (see Table 2.3). Higher total diurnal cortisol and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol. Step 2 did not significantly improve the model ($p > .050$), and the total model remained significant, $F(3, 101) = 9.26$, $p < .001$, $R^2 = .22$ (see Table 2.3).

In the second hierarchical linear regression analysis, cortisol stress reactivity was predicted from the two indices of the cortisol circadian rhythm ($AUC_{diurnal}$ and diurnal cortisol decline) in Step 1, and their interaction in Step 2. No significant effects were found, all *p's* > .050 (see Table 2.3).

Table 2.3 | *Results from Regressions Predicting Total Stress Cortisol and Cortisol Stress Reactivity from the Indices of the Cortisol Circadian Rhythm*

	Model 1		Model 2	
	B	*ß*	*B*	*ß*
Total stress cortisol				
Step 1				
Total diurnal cortisol	< 0.01	.59***	< 0.01	.56***
Diurnal cortisol decline	-0.01	-.25*	-0.01	-.25*
Step 2				
Total diurnal cortisol x Diurnal cortisol decline			< 0.01	.05
R^2_{change}	.21***		< .01	
R^2_{model}	.21***		.22***	
Cortisol stress reactivity				
Step 1				
Total diurnal cortisol	< -0.01	-.02	< -0.01	-.02
Diurnal cortisol decline	< -0.01	-.09	< -0.01	-.09
Step 2				
Total diurnal cortisol x Diurnal cortisol decline			< 0.01	.01
R^2_{change}	.01		< .01	
R^2_{model}	.01		.01	

Note. No outliers were removed because Cook's distances indicated no potentially influential data points.
* $p < .050$, *** $p < .001$.

2.4 Discussion

This study investigated associations between the cortisol circadian rhythm and cortisol stress responses to a social evaluative stress test in normally developing 6-year-olds. The laboratory stress test was effective in producing a significant rise in cortisol in the children. Higher total diurnal cortisol and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol. Together, these indices of the cortisol circadian rhythm explained 21.0% of the variance of total stress cortisol. No associations between the cortisol circadian rhythm indices and the cortisol stress reactivity index were found.

The positive association between total diurnal cortisol ($AUC_{diurnal}$) and total stress cortisol (AUC_{stress}) suggests that a generally higher circadian baseline cortisol production is associated with secreting more cortisol in stressful situations. The HPA-axis may be generally more active and/or both aspects of cortisol production may facilitate each other. As the total AUC_{stress} measure represents the cortisol concentration of basal/anticipatory, response, and recovery periods together, this might mean that children with generally higher total diurnal cortisol concentrations are more physiologically stressed by the entire laboratory procedure, instead of only by the stress-inducing components. That no associations between the cortisol circadian indices and the cortisol stress reactivity index were found may support this idea. Alternatively, given that the CREST was carried out during a school day, children with higher total diurnal cortisol may generally have elevated cortisol concentrations at school, or even more generally in daily life and not in response to the stress test procedure *per se*.

A potential underlying mechanism explaining the above described cortisol production patterns may be self-regulation. Higher self-regulatory capacities allow children to control their emotions, behavior and the stressfulness of events, and may result in lower cortisol stress concentrations and faster recovery during stressful situations, as well as during the day in general. Support for this idea comes from a study by Watamura et al. (2004) who found a negative association between effortful control (which is supported by selfregulatory capacities) and overall diurnal cortisol concentrations in 12- to 36-month-olds.

The negative association between diurnal cortisol decline and AUC_{stress} indicates that children with a smaller diurnal decline had higher cortisol concentrations in the stressful situation. Previous research has linked a smaller diurnal decline in children to lower levels of maternal parenting quality (involvement and warmth; Pendry & Adam, 2007), and more parent-child conflict at home (auditory assessments; Slatcher & Robles, 2012). Moreover, diurnal cortisol of foster children following an intensive family-based therapeutic intervention became more normative over time whereas the diurnal declines of

foster children not following this intervention became smaller over time (Fisher, Stoolmiller, Gunnar, & Burraston, 2007). Regarding cortisol stress responses, Bernard and Dozier (2010) found that in 11- to 20-month-olds, a disorganized attachment style, often seen as a reflection of lower quality maternal care, was associated with a cortisol increase in response to a stressor. Children with an organized attachment style did not show a cortisol increase in response to this situation. Additionally, in adults, attachment anxiety was positively associated with the cortisol response to an acute stressor (Quirin, Pruessner, & Kuhl, 2008). This may suggest that lower parenting quality may underlie a smaller diurnal decline and heightened total stress cortisol, potentially via mechanisms such as early life stress and/or reduced parental scaffolding during the development of selfregulation.

We found no associations between the cortisol circadian rhythm and the cortisol stress reactivity index in our study. In line with this, Kidd et al. (2014) also found no association between diurnal cortisol decline and the magnitude of the cortisol response to a stressor in older adults (between the ages of 54 and 76). Moreover, van Eck et al. (1996) found no association between (basal) diurnal cortisol concentrations and the cortisol stress response to a laboratory stressor in 27- to 57-year-olds. Although this is speculative and more research is needed, our results may add to this that also in 6-year-olds diurnal HPA-axis activity may not be associated with cortisol stress reactivity. This may suggest that in young children responding with increased cortisol to an acute stressor, with the goal of mobilizing energy to cope with the stressor (e.g., Nicolson, 2007), is independent of diurnal HPA-axis activity. Cortisol stress reactivity may be predicted (more strongly) by other factors, such as the early life environment, genetic factors, or the type of stressful situation (e.g., de Weerth, Buitelaar, & Beijers, 2013b; Dickerson & Kemeny, 2004; Gunnar, Talge, & Herrera, 2009; Kudielka, Schommer, Hellhammer, & Kirschbaum, 2004; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011).

However, Kidd et al. (2014) did find a positive association between total diurnal cortisol concentrations and the magnitude of the cortisol stress response. Additionally, Koss et al. (2016) found that blunted diurnal decline was associated with a blunted cortisol response toward a laboratory challenge in a group of predominantly adopted children. Differences between the study populations may at least in part underlie the differences in results. Koss et al. (2016) studied children that may be assumed to have had harsh early life environments (i.e., post-institutionalized and foster children), while our children came from a normal middle class sample background. The severe early life stress that is associated with harsh environments is known to affect the development of the HPA-axis (e.g., Loman & Gunnar, 2010; Lupien et al., 2009; Kudielka et al., 2009), potentially also affecting the dynamics of HPA-axis functioning. Differences between our findings and those of Kidd et al. (2014) might be explained by the age of the study populations: older adults

vs. children in whom the cortisol circadian rhythm still appears to develop (Shirtcliff et al., 2012; Simons et al., 2015). However, also other study characteristics, such as morning-afternoon differences, may explain our results. Supporting this idea, Kudielka et al. (2004) found that higher basal saliva cortisol concentrations were associated with lower stress-related net increases in saliva cortisol in the morning but not in the afternoon, when analyzing both separately. Contrary to our study, Kidd et al. (2014) collected cortisol stress response data both in morning and afternoon hours, possibly explaining the differences in findings. Examining HPA-axis functioning at various time points, at different ages, and in normative as well as clinically relevant populations, will help to further understand the associations between the cortisol circadian rhythm and cortisol stress responses.

Finally, we did not find support for associations of the interaction between the two indices of the cortisol circadian rhythm on cortisol stress responses. This may suggest that the indices of the cortisol circadian rhythm do not further facilitate each other's individual effects, but more research is needed to further explore this.

2.4.1 Strengths, Limitations, and Future Research

Strengths of the current study are the relatively large sample size and early age of the participants. Another strength is the use of an innovative and effective social evaluative stress test that was especially designed to induce cortisol stress responses at this specific age (de Weerth et al., 2013a). Moreover, the combination of several indices of both the cortisol circadian rhythm as well as the cortisol stress response provides insight in the dynamics of HPA-axis functioning. A limitation of the current study is the fact that the cortisol stress responses were measured on a week/school day, whereas the cortisol circadian rhythm was measured during weekend days. This may have decreased associations between the two aspects of HPA-axis functioning making our findings an underestimation of the real effects. Additionally, we did not assess the cortisol awakening response (CAR), another index of the cortisol circadian rhythm (e.g., Nicolson, 2007), because mothers of 6-year-olds may often miss their child's exact awakening time, resulting in unreliable CAR assessments. However, including a reliable measure of this index (e.g., by using actigraphy to determine time of awakening) may potentially have resulted in the ability to explain more variance of the cortisol stress responses. Finally, in this study causality cannot be inferred from the results, since inverse relations between cortisol stress responses and circadian cortisol cannot be excluded.

Since in childhood and adolescence alterations in both the cortisol circadian rhythm and the cortisol stress response are associated with physical and psychological health (e.g., Buske-Kirschbaum et al., 2003; Carrion et al., 2002; Hankin et al., 2010; Luby et al., 2003; Ruttle et al., 2013; Shirtcliff & Essex, 2008; Watamura, Coe, Laudenslager, &

Robertson, 2010; White, Gunnar, Larson, Donzella, & Barr, 2000), a question for future research is whether alterations in the *associations* between the two aspects of cortisol production are linked to behavioral functioning, syndromes and illnesses. Moreover, since the cortisol circadian rhythm continues to develop during childhood and adolescence (e.g., Shirtcliff et al., 2012; Simons et al., 2015) a future question is how associations between these two patterns of HPA-axis functioning change with age. Finally, in this sample large inter-individual differences were found in cortisol responses to the stress test (e.g., 54.9% of the children showed an increase in cortisol from basal to peak response concentrations while the rest showed no change or a decrease). In future studies it would be interesting to further explore these inter-individual differences, for example, by determining whether they are explained by additional correlates such as stress early in life and/or genetic factors.

2.4.2 Conclusion

In this study, higher total diurnal cortisol and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol concentrations in normally developing 6-year-olds. Possible explanations for the patterns found are links with children's self-regulatory capacities and parenting quality.

Chapter 3

Development of the Cortisol Circadian Rhythm in the Light of Stress Early in Life

Based on:

Simons, S. S. H., Beijers, R., Cillessen, A. H. N., & de Weerth, C. (2015). Development of the cortisol circadian rhythm in the light of stress early in life. *Psychoneuroendocrinology, 62*, 292-300. doi:10.1016/j.psyneuen.2015.08.024

Abstract

The secretion of the stress hormone cortisol follows a diurnal circadian rhythm. There are indications that this rhythm is affected by stress early in life. This paper addresses the development of the cortisol circadian rhythm between 1 and 6 years of age, and the role of maternal stress and anxiety early in the child's life on this (developing) rhythm. Participants were 193 healthy mother-child dyads from a community sample. Self-reported maternal stress and anxiety and physiological stress (saliva cortisol), were assessed prenatally (gestational week 37). Postnatally, self-reported maternal stress and anxiety were measured at 3, 6, 12, 30, and 72 months. Saliva cortisol samples from the children were collected on two days (four times each day) at 12, 30, and 72 months of age. The total amount of cortisol during the day and the cortisol decline over the day were determined to indicate children's cortisol circadian rhythm. Multilevel analyses showed that the total amount of cortisol decreased between 1 and 6 years. Furthermore, more maternal pregnancy-specific stress was related to higher total amounts of cortisol in the child. Higher levels of early postnatal maternal anxiety were associated with flatter cortisol declines in children. Higher levels of early postnatal maternal daily hassles were associated with steeper child cortisol declines over the day. These results indicated developmental change in children's cortisol secretion from 1 to 6 years and associations between maternal stress and anxiety early in children's lives and children's cortisol circadian rhythm in early childhood.

***Keywords**:* cortisol circadian rhythm, maternal stress, maternal anxiety, prenatal, early life, child development

3.1 Introduction

Cortisol is the primary hormonal end product of the hypothalamic-pituitary-adrenal (HPA) axis, a major player in the human stress system. Cortisol is secreted in a pulsatile fashion throughout the day and follows a well-defined circadian rhythm. This is characterized by an early morning peak followed by a gradual decline throughout the day - steepest in the first three hours after awakening - reaching its lowest values around midnight (Edwards, Clow, Evans, & Hucklebridge, 2001; Kirschbaum & Hellhammer, 1989). Two often-used markers of the cortisol circadian rhythm are the total amount of cortisol during the day (area under the curve; AUC) and the cortisol decline throughout the day (e.g., Saridjan et al., 2010; Watamura, Donzella, Kertes, & Gunnar, 2004). Abnormalities in the cortisol circadian rhythm are associated with (mental) health and behavioral problems in childhood and adolescence (e.g., Jessop & Turner-Cobb, 2008; Shirtcliff & Essex, 2008). However, relatively little is known about the topic of the present study: the longitudinal development of the cortisol circadian rhythm in early childhood.

3.1.1 Development of the Cortisol Circadian Rhythm

While infants are able to produce cortisol at birth, production does not follow a set pattern across the day, but rather a two-phase pattern, with peaks unrelated to particular periods of the day (Spangler, 1991). During the first year of life infants acquire the normative cortisol circadian rhythm (e.g., Custodio et al., 2007; de Weerth & van Geert, 2002; de Weerth, Zijl, & Buitelaar, 2003; Spangler, 1991). This development seems to parallel the development of the 24 h sleep-wake cycle (e.g., de Weerth et al., 2003; Spangler, 1991).

Cross-sectional studies further suggest that the cortisol circadian rhythm continues to develop after the first year of life. For example, total cortisol concentrations decrease between 12 and 20 months (Saridjan et al., 2010), and are higher in 12-, 18-, and 24-months-olds than in 30- and 36-month-olds (Watamura et al., 2004). These two studies also found that the cortisol decline over the day became flatter between infancy (12 months) and toddlerhood (36 months; Saridjan et al., 2010; Watamura et al., 2004). The decrease in total cortisol might parallel the development of self-regulation. Self-regulatory capacities increase in early childhood (e.g., Kochanska, Murray, & Harlan, 2000; Watamura et al., 2004) and have been associated with lower overall cortisol concentrations in 12- to 36-months olds (Watamura et al., 2004). However, although the development of self-regulation and the sleep-wake cycle continue until at least middle childhood (e.g., Crabtree & Williams, 2009; Raffaelli, Crockett, & Shen, 2005) and the cortisol circadian rhythm still develops between 9 and 15 years of age (see Shirtcliff et

al., 2012), there is a gap in what is known about the longitudinal development of the cortisol circadian rhythm during childhood.

Hence, the first aim of this study was to longitudinally investigate how the cortisol circadian rhythm develops from ages 1 to 6. We expected cortisol decline over the day to become more normative, i.e., to become steeper, towards age 6. Furthermore, we expected the total amount of daily cortisol to decrease with age.

3.1.2 Stress in Early Life and the Development of the Cortisol Circadian Rhythm

There are large inter-individual differences in the cortisol circadian rhythm in adults (e.g., Karlamangla, Friedman, Seeman, Stawksi, & Almeida, 2013). These differences have been associated, amongst other things, with environment-related factors such as childhood maltreatment (e.g., van der Vegt, van der Ende, Kirschbaum, Verhulst, & Tiemeier, 2009). Potentially, the early life environment, both prenatal (de Weerth & Buitelaar, 2005a) and postnatal (Loman & Gunnar, 2010) might affect the development of the child's cortisol circadian rhythm.

Fetal programming mechanisms have long-term effects on offspring development in animal models, and could explain how maternal prenatal stress and anxiety may affect the child's developing cortisol circadian rhythm (Beijers, Buitelaar, & de Weerth, 2014). Earlier studies support this idea. E.g., maternal prenatal stress and anxiety are positively associated with cortisol concentrations in 5-year-olds (Gutteling, de Weerth, & Buitelaar, 2005) and maternal prenatal anxiety is associated with flatter declines during the day in adolescence (O'Donnell et al., 2013; van den Bergh, van Calster, Smits, van Huffel, & Lagae, 2008). Potential fetal programming effects on the child's cortisol circadian rhythm may occur through various mechanisms, such as maternal cortisol concentrations, health-related behaviors, the immune system, or placental functioning (Beijers et al., 2014). However, the associations between maternal prenatal stress and anxiety and the developing cortisol circadian rhythm in early childhood have not yet been studied.

Regarding the postnatal period, earlier research suggested an association between maternal postnatal stress and anxiety and the child's cortisol stress system. For example, 12- to 20-month-olds of mothers with high levels of parenting stress had higher total cortisol concentrations than children of mothers without parenting stress (Saridjan et al., 2010). And 4- and 9-month-old children of mothers with anxiety disorders appeared to have higher baseline cortisol concentrations than children of non-anxious mothers (Feldman et al., 2009; Warren et al., 2003). Maternal postnatal stress and anxiety may affect (the development of) children's HPA-axis through cortisol in breast milk (Grey, Davis, Sandman, & Glynn, 2013; Hinde et al., 2015) or through maternal behavior. For example, maternal anxiety disorders are associated with differences in parenting behavior, such

as reduced sensitivity and responsivity and differences in parenting related to sleep. Furthermore, maternal feelings of stress, such as parenting stress or daily hassles, are for instance associated with more maternal withdrawal, stricter discipline, and less mother-child book reading (Anthony et al., 2005; Feldman et al., 2009; Karrass, VanDeventer, & Braungart-Rieker, 2003; Nicol-Harper, Harvey, & Stein, 2007; Repetti & Wood, 1997; Warren et al., 2003). As early life caregiving may profoundly impact the development of children's stress system (Loman & Gunnar, 2010), it is surprising that little attention has been paid to the association between maternal stress and anxiety early in the child's postnatal life and the developing cortisol circadian rhythm in early childhood.

Therefore, the second aim of this study was to investigate whether stress early in the child's life, as indicated by maternal prenatal and early (first 6 months) postnatal stress and anxiety predicted individual differences in children's cortisol circadian rhythm and its development. Prenatal and early postnatal maternal stress and anxiety were expected to be associated with an altered cortisol circadian rhythm (higher total cortisol concentrations and flatter declines during the day). Associations between prenatal and early postnatal maternal stress and anxiety, and the *development* of the cortisol circadian rhythm in early childhood were explored.

3.2 Methods

3.2.1 Participants

This study was part of an ongoing longitudinal project on psychobiological development in children (BIBO project; Basal Influences on Child Development). The project was approved by the Institutional Ethical Committee, which follows the Helsinki Declaration. Participating mothers signed informed consents. Pregnant women were recruited through midwife practices in and around the cities of Nijmegen and Arnhem, The Netherlands. Inclusion criteria were Dutch language fluency, no drug use during pregnancy, no physical or mental health problems, an uncomplicated singleton pregnancy with a term delivery, and a 5 min infant Apgar score of 7 or higher (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2010; Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011a). Participants in the total project were 220 healthy born children and their mothers, of whom 193 dyads were still in the project 3 months after delivery (see Beijers et al., 2011a). Of the 193 mothers, 94.3% was born in the Netherlands, 96.9% lived together with their partner, and 81.3% were employed during pregnancy. Mothers were between 21.10 and 42.90 years old at delivery (M_{age} = 32.46; *SD* = 3.80). The current study used data collected during one prenatal (37th week of pregnancy) and five postnatal measurements (3, 6, 12, 30, and 72 months). Table 3.1 shows the descriptive statistics for each measure (outliers removed).

3.2.2 Procedure

At week 37 of pregnancy mothers were asked to fill out questionnaires on general and pregnancy-specific stress and anxiety and were asked to collect circadian saliva samples. At the five postnatal measurement times, mothers were asked to complete questionnaires about their own feelings of general stress and anxiety. When the child was 12, 30, and 72 months old, mothers were asked to collect circadian saliva samples of their child.

3.2.3 Measures

3.2.3.1 Cortisol sampling

As part of the prenatal measurement, mothers (*n* = 163) collected saliva samples on two consecutive weekdays (87.7%: two consecutive days; 89.0%: two days within one week) at 5 predefined times: immediately after awakening (C1), 30 min after awakening (C2) and at 12:00 (C3), 16:00 (C4), and 21:00 (C5). At the 12, 30, and 72 month measurement moments mothers (*n* = 162, 161, 148, respectively) collected 8 saliva samples of their child on two preferably consecutive days (72.0%: two consecutive days; 87.0%: two days within one week) at 4 predefined times: immediately after the child had woken up (C1) and at 11:00 (C2), 15:00 (C3), and 19:00 (C4). Mothers were instructed to collect these samples on days that the child did not attend childcare or school.

All child saliva samples were collected using eye sponges as saliva sampling devices (BD Visispeare, Waltham, MA; de Weerth, Jansen, Vos, Maitimu, & Lentjes, 2007). Mothers were asked to collect their own saliva through direct spitting. Participants were asked to store all collected saliva samples in their home freezer until transported to the university. At the university, the eye sponges were centrifuged for 10 min at 3948g, and the extracted saliva was then stored in a freezer (-25 °C). Subsequently, samples were analyzed by the Laboratory of Endocrinology, University Medical Center Utrecht using an in-house competitive radio-immunoassay employing a polyclonal anticortisol-antibody (K7348) and [1,2-^{3}H(N)]-Hydrocortisone (PerkinElmer NET396250UC) as a tracer. The lower limit of detection was 1.0 nmol/L and inter-assay and intra-assay variations were < 10.0%.

3.2.3.2 Anxiety

Maternal state anxiety was measured using the 20-item state subscale of the State-Trait Anxiety Inventory (STAI; Cronbach's α 0.90-0.96; Spielberger, 1983; van der Ploeg, Defares, & Spielberger, 1981; 0.91-0.93 in our sample). Mothers rated how much each item applied to them on a 4-point scale (1 = *not at all*, 4 = *very much*). A higher total score represents a higher level of state anxiety. This measure was collected prenatally and at each of the five postnatal times.

3.2.3.3 Stress

Daily hassles were measured using the 49-item Alledaagse Problemen Lijst (APL; test-retest reliabilities 0.76-0.87; Vingerhoets, Jeninga, & Menges, 1989). Each item describes one event. Participants indicated for each event whether it had occurred in the last 2 months, and, if so, rated how much it had bothered them on a 4-point scale (1 = *not at all,* 4 = *very much*). A mean intensity rating was calculated for each participant by dividing the sum of these ratings by the number of reported events. Higher values indicated more experienced negativity as a result of daily hassles. This measure was collected prenatally and at 3, 6, and 12 months postnatally.

3.2.3.4 Pregnancy-specific anxiety

Pregnancy-specific anxiety was measured using two subscales of the Pregnancy-specific Anxiety Questionnaire-Revised (PRAQ-R; Buitelaar, Huizink, Mulder, Robles de Medina, & Visser, 2003; Huizink, Mulder, & Buitelaar, 2004a; Huizink, Robles de Medina, Mulder, Visser, & Buitelaar, 2003). These subscales measure fear of giving birth (3 items; Cronbach's α 0.79-0.83; Huizink, Mulder, Robles de Medina, Visser, & Buitelaar, 2004b; 0.70 in our sample) and fear of bearing a child with disabilities (4 items; Cronbach's α 0.87-0.88; Huizink et al., 2004b; 0.83 in our sample). Items were rated on a 5-point scale (1 = *not at all applicable*, 5 = *very much applicable*). Higher scores indicated higher levels of pregnancy-specific anxiety.

3.2.3.5 Pregnancy-specific stress

Pregnancy-specific daily hassles were measured using the 43-item Pregnancy Experience Scale (PES; Cronbach's α 0.91-0.95; DiPietro, Ghera, Costigan, & Hawkins, 2004; in our sample 0.87 for positive items and 0.88 for negative items). Each item describes a pregnancy-specific experience. Participants rated to what degree each item resulted in a positive and in a negative experience on a 4-point scale (0 = *not at all*, 3 = *very much*). The ratio of negative to positive experiences was calculated (sum of the negative ratings divided by sum of the positive ratings). Higher scores indicated a more negative emotional valence towards pregnancy.

3.2.3.6 Demographics

Prenatally, mothers were asked to indicate their highest educational level from 9 options ranging from 1 *(primary)* to 8 *(university),* followed by the option 9 *(other)*. Answers on this last option (n = 2) were recoded into the closest matching option. After birth, gender of child and birth order (first child yes/no) were recorded.

3.2.3.7 Number of months a child was breastfed during its first year
Monthly maternal interviews during the first 12 months of the child's life provided information about breastfeeding (Beijers et al., 2010).

3.2.4 Data Preparation

3.2.4.1 Child cortisol
On two collection days during the 12 (*M* = 11.48, *SD* = 0.57, range 11.00-13.00 months), 30 (*M* = 29.57, *SD* = 1.05, range 28.00-36.50 months), and 72 month (*M* = 72.62, *SD* = 1.72, range 70.00-80.50 months) data collection moments, mothers (*n* = 162, 161, and 148, respectively) collected a total of 3484 saliva samples from their children. Of these samples, 3357 were analyzable (96.3%). To decrease fluctuations in cortisol due to sampling time, samples taken within the following time ranges were accepted: C1 between 6:00 and 10:00 (Beijers et al., 2010), C2 between 10:00 and 12:00, C3 between 14:00 and 16:00 (two hour window; Tollenaar, Jansen, Beijers, Riksen-Walraven, & de Weerth, 2010), and C4 between 18:00 and 21:00. The window of 2 h was extended with one hour for C4 (Beijers et al., 2010) since diurnal cortisol fluctuation is less extreme at the end of the day (Edwards et al., 2001; Kirschbaum & Hellhammer, 1989). Illnesses, the use of medication, and biologically extreme values were also taken into account. In total, 278 of the analyzable samples were eliminated (8.3%). The remaining samples were used to calculate the child's total amount of cortisol during the day, that is, the area under the curve to the ground (AUCg; Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003) and the cortisol decline from morning to evening (decline or slope: C1 minus C4). Both measures (AUCg and decline) were first calculated per day and then averaged across days (Watamura et al., 2004). Of the 193 children, 18 dropped out of both main (multilevel) analyses since they did not have any of the decline or AUCg outcome measures. T-tests showed no significant differences between dropouts and children in the analyses on any of the prenatal and early postnatal (3 and 6 months) predictors.

3.2.4.2 Maternal cortisol
On two collection days during the 37th week of pregnancy (*M* = 37 weeks, 0.72 days; *SD* = 9.37 days), 163 mothers collected a total of 1532 saliva samples. Of these samples, 1529 were analyzable (99.8%). To decrease fluctuations in these cortisol samples due to sampling time, the following time ranges were used for accepting samples: C1 between 6:00 and 10:00 and within 15 min after awakening, C2 between 25 and 35 min after awakening, C3 between 11:30 and 13:30, C4 between 15:30 and 17:30 and C5 between 20:00 and 23:00 (Beijers et al., 2010). Additionally, samples collected during/after the day of delivery were removed. In total, 98 of the analyzable samples were removed

(6.4%). From the remaining samples, cortisol decline from morning to evening (decline or slope: C1 minus C5), the total amount of cortisol during the day (AUCg using C1, C3, C4 and C5; Pruessner et al., 2003), the cortisol awakening response (CAR: C2 minus C1), and evening cortisol (C5) were calculated. Previous research has shown that the cortisol decline from morning to evening and evening cortisol measures are important markers of the maternal stress system in relation to child outcome variables (Beijers et al., 2010). Therefore, our main analyses were conducted with these two non-correlated variables (r = -.12, p > .050). Findings of supplementary analyses with the other measures (maternal AUCg and CAR) can be found in Footnote 3.2.

3.2.4.3 Early postnatal maternal stress and anxiety

A score for early postnatal maternal stress in the first half year of the child's life was computed as the average of the 3 and 6 month daily hassles scores (r = .62, p < .001). A score for early postnatal maternal anxiety in the first half year of the child's life was computed as the average of the 3 and 6 month anxiety scores (r = .54, p < .001).

3.2.5 Statistical Analyses

Correlations between the markers of the child cortisol circadian rhythm (total amount of cortisol and decline from morning to evening) on both sampling days within one measurement moment were calculated. To test the differences between these correlations, correlated contrast for independent correlations were used (Steiger, 1980). To be conservative, the n of the smallest cell in each correlation table was used in these calculations.

Subsequently, mean scores of the child cortisol markers per measurement round were calculated and all variables were checked for normality and outliers (3 *SD*) across the repeated measures (12, 30, and 72 months). The following outliers were detected: AUCg at 30 months (n = 2), cortisol decline from morning to evening at 30 months (n = 1), early postnatal maternal anxiety, mean 3 and 6 months (n = 1), maternal anxiety at 30 months (n = 1), maternal anxiety at 72 months (n = 1), maternal prenatal evening cortisol (n = 2). Since an advantage of multilevel analyses is its robustness for missing data, i.e., there is no requirement for complete data (Tabachnick & Fidell, 2007), outliers were removed before analyzing. To improve the normal distribution of the level one residuals, a square root transformation was used on the AUCg and pregnancy-specific stress scores. The variables used in our study were thereafter (approximately) normally distributed, according to the Kolmogorov-Smirnov test as well as visual inspection, across the repeated measures (12, 30, and 72 months).

To test how the circadian rhythm developed over time, longitudinal regression analyses using mixed-model (multilevel) designs were performed. In these analyses, the

cortisol measures of the children at 12, 30, and 72 months (mean decline and AUCg scores over Day 1 and 2) were used at Level 1 and nested within the children at Level 2. To examine whether the nested structure was required, the intraclass correlation (ICC) was calculated using a null model. The ICC for children's cortisol AUCg measure was .16, indicating that 16.0% of the variability in children's total cortisol concentration during the day was associated with differences between children, and that multilevel analyses were applicable. Multilevel analyses were also found applicable for children's morning to evening cortisol decline scores (ICC = .06).

Thereafter, a build-up strategy was followed in which variables were added one-by-one to the model with random intercept (allowing the intercept of the regression line to vary per participant). After adding each variable the change in deviance on the -2 log likelihood ratio scale after generalized least square estimations was assessed. Variables that did not improve the model by significantly reducing the deviance were excluded. Time (considered as a random factor, allowing the slope of the regression line to vary per participant) was entered into the model first. Time was weighted per spacing of the assessments in months and fractions of months. The time models with the best model fit are presented in the results (Section 3.3.2).

To test whether prenatal and early postnatal stress and anxiety of the mother predicted (the development of) the circadian rhythm between age 1 and 6 the following build-up strategy was used. Firstly, confounders were entered into the best fitting time models (Section 3.3.2), followed by the predictors, the prenatal and postnatal maternal stress and anxiety measures, as well as the interactions between time and the maternal stress and anxiety variables (prenatal and early postnatal). To control for possible confounding factors, the following variables were taken into account: maternal education, child gender, birth order (first child yes/no), the number of months a child was breastfed during its first year, maternal anxiety during the 12, 30, and 72 month measurement moments, and maternal daily hassles during the 12 month measurement (not measured during the 30 and 72 month data collection). The time distances between the morning (C1) and evening (C4) cortisol samples of the child were also used as confounders in the models. The best fitting models were obtained by adding each predictor one-by-one and excluding predictors that did not result in a statistical improvement of the models (e.g., Beijers et al., 2011a; de Weerth, Buitelaar, & Beijers, 2013b). The models with the best fit are presented in the results (Section 3.3.3).

3.3 Results

3.3.1 Preliminary Analyses

Correlations were calculated between the cortisol measures representative of two different sampling days at 12, 30, and 72 months. The total amount of cortisol during the day (AUCg) on Day 1 and 2 correlated (marginally) significantly with each other at each time point (r = .38, p = .003, r = .21, p = .066, and r = .58, p < .001 at 12, 30, and 72 months, respectively). Correlated contrasts indicated no significant difference between the correlations at 12 and 30 months (z = -0.90, p = *ns*). Correlations at 30 and 72 months differed significantly from each other (z = 2.20, p = .028). Hence, day-to-day stability of the total amount of cortisol during the day (AUCg) seemed to increase between 30 and 72 months of age. Cortisol decline from morning to evening also correlated significantly between Day 1 and Day 2 at each time point (r's = .27, .22, and .30, at 12, 30, and 72 months respectively, all p's < .050). These correlations were not significantly different from each other.

The (marginally) significant correlations between the cortisol measures on the two days within one data collection round allowed us to calculate means of these measures for each measurement moment. Descriptive statistics of these and the other untransformed variables are presented in Table 3.1 (outliers removed).

Correlations between the mean cortisol variables of the child (total amount of cortisol and cortisol decline from morning to evening) per measurement moment and maternal prenatal and early postnatal stress and anxiety variables were calculated (Table 3.2). Regarding correlations between predictor and child cortisol variables, higher postnatal maternal anxiety early in the child's life was associated with a flatter cortisol decline from morning to evening at 30 months of age (r = -.23, p = .008). Also, more postnatal maternal daily hassles early in the child's life were associated with higher total cortisol concentrations during the day at 72 months (r = .23, p = .013).

3.3.2 Development of the Cortisol Circadian Rhythm[3.1]

Tables 3.3 and 3.4 (see model 1) represent the multilevel models with time as predictor of the cortisol measures. The time effect was significant for the total amount of cortisol during the day (AUCg; Table 3.3, model 1; p < .001). The total amount of cortisol during the day decreased between 1 and 6 years of age.

[3.1] | Repeating the multilevel analyses with the child area under the curve with respect to increase (AUCi; Pruessner et al., 2003) instead of the morning evening difference as outcome measure resulted in similar outcomes with the same significant effects of early postnatal maternal anxiety (*Estimate* = 79.51, p = .035) and early postnatal maternal daily hassles (*Estimate* = -2262.41, p = .003).

Table 3.1 | *Descriptive Statistics of Child Cortisol, Confounding, and Predicting Variables (Untransformed Data)*

	N	*M*	*SD*	*Min*	*Max*
Confounding variables					
Child gender (% girls)	193	47.7%			
First born child (% yes)	193	41.5%			
Maternal education	185	6.64	1.52	1.00	8.00
Time difference between C1 and C4, at 12 months[a]	162	687.79	35.50	568.50	787.50
Time difference between C1 and C4, at 30 months[a]	161	686.58	32.28	587.50	772.00
Time difference between C1 and C4, at 72 months[a]	148	687.90	33.63	570.00	764.00
Anxiety at 12 months (STAI)	177	29.18	7.64	20.00	63.00
Anxiety at 30 months (STAI)	183	29.96	7.40	20.00	63.00
Anxiety at 72 months (STAI)	158	30.66	8.00	20.00	61.00
Daily hassles at 12 months (APL)	181	1.14	0.46	0.00	2.43
Breastfeeding (months in first year)	188	5.44	4.30	0.00	12.00
Prenatal factors					
Daily hassles (APL)	174	1.14	0.46	0.00	2.54
Anxiety (STAI)	174	32.20	8.88	20.00	64.00
Pregnancy-specific hassles (PES)	174	0.33	0.23	0.00	1.43
Fear of giving birth (PRAQ-R)	174	5.36	2.48	3.00	15.00
Fear of bearing a handicapped child (PRAQ-R)	174	9.17	3.37	4.00	20.00
Maternal cortisol decline (nmol/L)	149	6.74	4.47	-2.80	24.00
Maternal evening cortisol (nmol/L)	155	9.44	2.75	0.85	20.00
Early postnatal factors					
Anxiety, mean 3 and 6 months (STAI)	190	28.37	6.41	17.50	51.00
Daily hassles, mean 3 and 6 months (APL)	191	1.12	0.38	0.00	2.28
Child cortisol					
Cortisol AUCg at 12 months (nmol/L)	118	5944.19	1858.83	3057.50	11270.50
Cortisol AUCg at 30 months (nmol/L)	116	5092.65	1550.85	2710.50	8917.50
Cortisol AUCg at 72 months (nmol/L)	116	4828.50	1358.82	2518.25	10801.13
Cortisol decline at 12 months (nmol/L)	136	11.65	6.19	-9.70	27.95
Cortisol decline at 30 months (nmol/L)	136	13.36	6.05	-9.90	33.40
Cortisol decline at 72 months (nmol/L)	132	12.89	5.01	-0.35	26.00

Note. N's of physiological stress measures of the child are lower since more cortisol samples (collected according to the sampling rules described under 3.2.4.1) are needed to calculate the cortisol measures. E.g., all 4 diurnal samples were required to calculate the area under the curve to the ground (AUCg; Pruessner et al., 2003). STAI = state-trait anxiety inventory, APL = alledaagse problemen lijst/Dutch daily hassles questionnaire, PRAQ-R = pregnancy-specific anxiety questionnaire-revised, PES = pregnancy experience scale.

[a]Calculated in minutes.

Table 3.2 | *Pearson Correlations of Predictor and Outcome Variables*

	1[a].	2.	3.	4[a].	5[a].	6.	7.	8[a].	9.	10.	11.	12.	13.	14.
Prenatal														
1. Anxiety (STAI)[a]														
2. Daily hassles (APL)	.26***													
3. Pregnancy-specific hassles (PES)	.44***	.24**												
4. Fear of giving birth (PRAQ-R)[a]	.35***	.15^{+}	.28***											
5. Fear of bearing a handicapped child (PRAQ-R)[a]	.13^{+}	.10	.20**	.14^{+}										
6. Maternal cortisol decline	-.02	.01	.17*	-.04	-.18*									
7. Maternal cortisol, evening	-.02	-.05	-.18*	.06	-.04	-.12								
Postnatal														
8. Anxiety, mean 3 and 6 months (STAI)[a]	.54***	.19*	.35***	.37***	.15*	-.01	-.04							
9. Daily hassles, mean 3 and 6 months (APL)	.26**	.56***	.43***	.18*	.13^{+}	.05	-.08	.35***						
10. Cortisol AUCg at 12 months	-.00	.01	.14	-.02	.14	.08	.05	-.01	.09					
11. Cortisol AUCg at 30 months	.01	.19^{+}	.11	-.00	.09	.13	-.12	-.17^{+}	.06	.14				
12. Cortisol AUCg at 72 months	.08	.10	.16^{+}	-.05	-.08	.18^{+}	-.14	-.02	.23*	.15	.47***			
13. Cortisol decline at 12 months	-.14	-.06	-.07	-.00	.13	-.05	-.09	-.11	.10	.24*	-.09	-.08		
14. Cortisol decline at 30 months	-.12	-.12	-.13	-.08	-.14	.11	-.06	-.23**	-.08	.03	.40***	.18^{+}	.03	
15. Cortisol decline at 72 months	.08	-.02	.02	-.05	-.00	.12	-.04	-.04	.10	.18	.23*	.66***	-.01	.19^{+}

Note. STAI = state-trait anxiety inventory, APL = alledaagse problemen lijst/Dutch daily hassles questionnaire, PRAQ-R = pregnancy-specific anxiety questionnaire–revised, PES = pregnancy experience scale, AUCg = area under the curve to the ground.

[a]Spearman correlations (variable non-normally distributed).

$^{+}$ $p < .100$, *$p < .050$, ** $p < .010$, *** $p < .001$.

Table 3.3 | *Estimates for the Best Fitting Multilevel Models for the Total Cortisol Concentration (AUCg)*

	Cortisol AUCg		
	Estimate	*SE*	*p*
Model 1			
Intercept	75.95	.92	< .001***
Time	-.10	.02	< .001***
Deviance	2592.18		
Model 2			
Fixed effects			
Intercept	13.40	11.64	.251
Time	-.10	.02	< .001***
Time difference between C1 and C4, at 12, 30, 72 months[a]	.09	.02	< .001***
Maternal educational level	.24	.45	.590
Anxiety, at 12, 30, 72 months (STAI)	-.19	.10	.076[+]
Daily hassles at 12 months (APL)	1.54	1.41	.278
Maternal prenatal cortisol, decline	.12	.15	.392
Pregnancy-specific hassles (PES)	9.20	3.66	.013*
Anxiety, mean 3 and 6 months (STAI)	< .01	.11	.981
Deviance	1894.33		

Note. AUCg = area under the curve to the ground, STAI = state-trait anxiety inventory, APL = alledaagse problemen Lijst/Dutch daily hassles questionnaire, PES = pregnancy experience scale.
Excluded variables: child gender, first born child, breastfeeding, prenatal daily hassles (APL), prenatal anxiety (STAI), fear of giving birth (PRAQ-R), fear of bearing a handicapped child (PRAQ-R), prenatal maternal evening cortisol (nmol/L), early postnatal stress (APL, mean 3 and 6 months), the interactions between time and prenatal, as well as early postnatal stress and anxiety.
Models including or winsorizing outliers provided similar results.
[a]Calculated in minutes.
[+] $p < .100$, * $p < .050$, *** $p < .001$.

3.3.3 Stress in Early Life and the Development of the Cortisol Circadian Rhythm[3.2]

Models 2 in Tables 3.3 and 3.4 represent the best fitting multilevel models predicting the development of the child cortisol variables, with time, maternal stress and anxiety (prenatal and early postnatal), as well as confounders, and the interactions between time and maternal stress and anxiety (prenatal and early postnatal) as predictors. The model predicting the total amount of cortisol during the day (AUCg; see Table 3.3, model 2) indicated that it was

[3.2] | Multilevel models using the maternal prenatal total amount of cortisol (AUCg) or the maternal prenatal cortisol awakening response (CAR) as predictors of the markers of the child cortisol circadian rhythm (AUCg and Decline) indicated that these maternal prenatal cortisol measures did not significantly predict the child cortisol variables.

predicted by time ($p < .001$) and maternal pregnancy-specific hassles ($p = .013$). The total amount of cortisol during the day decreased between 1 and 6 years of age and higher levels of pregnancy-specific hassles were associated with higher total cortisol concentrations.

Regarding the cortisol decline, Table 3.4 (model 2) shows that higher levels of early postnatal maternal anxiety during the first 6 months of the child's life were associated with a flatter cortisol decline during the day (less decline from morning to evening, $p = .024$), and more postnatal maternal daily hassles in the first 6 months of the child's life were associated with a steeper cortisol decline during the day (more decline from morning to evening, $p = .024$).

Table 3.4 | *Estimates for the Best Fitting Multilevel Models for Cortisol Decline from Morning to Evening*

	Cortisol decline		
	Estimate	*SE*	*p*
Model 1			
Intercept	12.11	.51	< .001***
Time	.01	.01	.227
Deviance	2564.77		
Model 2			
Fixed effects			
Intercept	-7.27	6.50	.265
Time	.01	.01	.629
Time difference between C1 and C4, at 12, 30, 72 months[a]	.03	.01	.001**
Maternal educational level	.25	.24	.299
Breastfeeding	< -.01	.08	.981
Anxiety, at 12, 30, 72 months (STAI)	-.02	.06	.654
Daily hassles at 12 months (APL)	.14	.93	.877
Maternal prenatal cortisol, decline	.08	.08	.323
Prenatal daily hassles (APL)	-1.41	.94	.137
Anxiety, mean 3 and 6 months (STAI)	-.14	.06	.024*
Daily hassles, mean 3 and 6 months (APL)	2.75	1.22	.024*
Deviance	1882.93		

Note. STAI = state-trait anxiety inventory, APL = alledaagse problemen lijst/Dutch daily hassles questionnaire. Excluded variables: child gender, first born child, prenatal anxiety (STAI), pregnancy-specific hassles (PES), fear of giving birth (PRAQ-R), fear of bearing a handicapped child (PRAQ-R), prenatal maternal evening cortisol (nmol/L), the interactions between time and prenatal, as well as early postnatal stress and anxiety. Models including or winsorizing outliers provided similar results.

[a]Calculated in minutes.

* $p < .050$, ** $p < .010$, *** $p < .001$.

3.4 Discussion

3.4.1 Development of the Cortisol Circadian Rhythm

In the present study the total amount of cortisol during the day decreased between 1 and 6 years of age. This is in line with earlier research (Saridjan et al., 2010; Watamura et al., 2004) and may in part be related to children's increasing self-regulatory capacities (e.g., Kochanska et al., 2000; Raffaelli et al., 2005; Watamura et al., 2004). Possibly, increasing, self-regulation capacities enable children to better control their emotions, behavior, and the stressfulness of events. This would result in lower cortisol reactions to (daily) stressors, and in turn, in a lower total amount of cortisol during the day. The negative association between effortful control and overall cortisol concentrations in 12- to 36-month-old's found by Watamura et al. (2004) supports this idea.

Between the age of 1 and 6 years no change in the developmental trajectory of cortisol decline was found in our study. Contrary to this, Watamura et al. (2004) and Saridjan et al. (2010) found that the diurnal cortisol decline became flatter between 12 and 20 and 12-36 months. This difference might be explained by differences in saliva collection days. We sampled on non-childcare or non-schooldays whereas Saridjan et al. (2010) and Watamura et al. (2004) collected saliva on one weekday and two days within two weeks, respectively. Potentially, children in these studies provided samples more often on non-parental care or preschool days, especially as they became older. Children's cortisol levels seem to increase during the day from morning to afternoon in center-care, especially between 24 and 36 months (see Vermeer & van IJzendoorn, 2006). This might explain the finding that cortisol curves became flatter with age in the earlier studies. On days where the primary caregiver is available to aid the child's stress regulation cortisol might increase less than on days when the child attends childcare or goes to school and the primary caregiver is not present. The absence of the expected increase in steepness of the decline might suggest that infants around the age of 12 months have already fully developed the adult-like cortisol decline during the day. However, it could also be that development towards a steeper decline (also) takes place after the age of 6.

Interestingly, we found an increase in day-to-day stability of total cortisol concentrations between 30 and 72 months. This might be associated with children's sleeping habits. Young children often nap during the day, and during a nap cortisol levels appear to decline, rebounding after waking-up (Larson, Gunnar, & Hertsgaard, 1991). This causes fluctuations in cortisol samples taken mid-morning or mid-afternoon, when children usually take their nap. These fluctuations are less likely to occur for older children who nap less, resulting in higher stability of the AUCg. Also, the potential association between developing self-regulation and stress reactions described above might reduce day-to-day vari-

ability in cortisol. Together with the finding of low day-to-day stability in 3- to 7-month-olds (Spangler, 1991) and decreasing intra-individual variability at the end of the first year (Tollenaar et al., 2010), our results suggest that day-to-day stability continues to develop after the first year of life, possibly reflecting consolidation of the HPA-axis over time.

3.4.2 Stress in Early Life and the Development of the Cortisol Circadian Rhythm

Higher levels of maternal pregnancy-specific hassles were associated with higher total cortisol concentrations of the child. Higher levels of early postnatal maternal anxiety were associated with flatter child cortisol declines. Higher levels of early postnatal maternal daily hassles were associated with steeper child cortisol declines. These associations were not moderated by time, suggesting that they remained stable between 1 and 6 years of age.

3.4.2.1 Maternal prenatal stress and anxiety

Gutteling et al. (2005) found that higher maternal prenatal cortisol and pregnancy-specific *anxiety* were related to higher offspring cortisol concentrations at age 5. We add to this that pregnancy-specific *stress* is associated with higher child cortisol concentrations. A potential underlying mechanism is that pregnancy-specific stress may affect maternal health-related behavior including eating, sleep, and physical exercise (potentially reducing maternal immune system- and placental-functioning). This may in turn result in a less healthy, more stressful environment for the unborn child, affecting its HPA-axis (for a review, see Beijers et al., 2014). Support for this idea is found by Lobel et al. (2008) who indicated that pregnancy-specific stress is associated with less healthy behavior of the pregnant mother (including smoking, unhealthy eating, and less physical exercise), and a lower gestational age of the child.

The finding that especially pregnancy-specific hassles seem to play a role might be explained by the timing of our assessment. At 37 weeks of pregnancy, women in the Netherlands are on pregnancy leave and the physical strain of pregnancy is often large, possibly making pregnancy-specific hassles more salient. The timing may also explain why maternal *cortisol* did not predict child cortisol in our study (contrary to Gutteling et al., 2005). During the second and third trimester of pregnancy, maternal cortisol concentrations rise substantially (de Weerth & Buitelaar, 2005b). High maternal cortisol concentrations earlier in pregnancy (week 15-17, Gutteling et al., 2005), when cortisol concentrations should still be low may therefore have more impact on child development than high levels in late pregnancy.

We found no associations between prenatal anxiety and cortisol decline where O'Donnell et al. (2013) and van den Bergh et al. (2008) found that maternal prenatal anxiety predicted flatter cortisol declines in adolescents. Prenatal anxiety and stress may be associated with offspring cortisol concentrations differentely at different ages. Rat

models indicate that stress in early life (maternal separation) affects the HPA-axis differently at different ages (Workel et al., 2001). Furthemore, hormonal changes taking place in adolescence, or an accumulation of the anxiety-related effects over a more prolonged time might result in the effects showing up in adolescence.

3.4.2.2 Maternal postnatal stress and anxiety

The finding that higher levels of early postnatal maternal anxiety were associated with flatter cortisol declines from age 1 to 6 is in line with our hypothesis and earlier findings of associations between maternal anxiety disorders and higher baseline child cortisol concentrations (Feldman et al., 2009; Warren et al., 2003). Our association between early postnatal maternal anxiety (first 6 months) and flatter cortisol declines was found adjusting for later postnatal maternal anxiety (12, 30, and 72 months). This suggests that maternal anxiety especially early in the child's life plays a role in the development of the cortisol circadian rhythm. An important potential underlying mechanism may be maternal behavior. This is supported by studies relating maternal anxiety disorders with less optimal parenting, including lower sensitivity and responsivity (Feldman et al., 2009; Nicol-Harper et al., 2007; Warren et al., 2003), and by Loman and Gunnar (2010) who stated that in the first years of life stress related to caregiving experiences may play a fundamental role in the development of the child's stress system. While high quality care helps infants regulate stress and contributes to later regulatory capacities, low quality care may be an extra source of stress (Albers, Riksen-Walraven, Sweep, & de Weerth, 2008; Loman & Gunnar, 2010). Non-optimal parenting may thus mediate the link between maternal anxiety and the child's HPA-axis (see also Ben-Dat Fisher et al., 2007, who found that less supportive maternal care was associated with flatter cortisol declines in preschoolers). A related mechanism may be disturbance of the child's 24 h sleep-wake cycle. For example, Warren et al. (2003) found that infants of mothers with panic disorders had more disturbed sleep than control infants, potentially due to sleep-related parenting behavior (e.g., mothers with panic disorders more often feed the child at bedtime, sleep together with the infant, and less often put the infant to bed awake at 15-16 months).

The association between more postnatal maternal daily hassles early in the child's life and steeper child cortisol declines is contrary to our expectation and suggests that maternal stress, in the form of daily hassles, and anxiety are associated in opposite manners to the child's cortisol decline. Although the terms stress and anxiety are frequently used interchangeably, anxiety is typically viewed as having anticipatory and affective components, such as subjective feelings of tension, apprehension, and worry, while stress is most commonly viewed as a response to a present challenge (Berridge, 2005; Nolen-Hoeksema, 2006). Stress in the form of daily hassles refers specifically to the re-

sponse to the irritating, frustrating, and distressing demands of everyday life (Kanner, Coyne, Schaefer, & Lazarus, 1981). Stress, including daily hassles, and anxiety may thus have different effects on maternal behavior. While maternal anxiety disorders, as discussed above, are related to less optimal parenting, Karrass et al. (2003) found maternal daily hassles to be associated with less shared mother-child book reading, but not with parenting behavior in a mother-child interaction (e.g., sensitivity). Our conflicting findings were for maternal stress and anxiety during the first 6 postnatal months of life. This is a period in which most mothers are dealing with the challenges of caring for a new infant (e.g., establishing sleeping and feeding routines) and transitioning back to work, while at the same time many are often worrying about the infant's health and providing optimal caregiving. Hence, many mothers will be coping with both stress *and* anxiety. More research is needed to find out how early postnatal stress and anxiety differentially and in combination with each other affect maternal behavior, in turn possibly affecting the development of the offspring's HPA-axis in different manners.

Finally, a potential mediator linking both postnatal maternal stress and anxiety with the child's HPA-axis might be cortisol in breast milk. To date, little is known about associations between maternal psychological status and cortisol in breast milk. However, maternal milk cortisol concentrations have been associated with offspring temperament in humans (Grey et al., 2013) and rhesus monkeys (Hinde et al., 2015). Theoretically, programming effects of cortisol in breast milk could influence the development of the offspring's HPA-axis, leading also to differences in temperament.

Summarizing, pregnancy-specific stress and early postnatal maternal stress and anxiety seem to be associated with the child's cortisol circadian rhythm from age 1 to 6. Different prenatal and postnatal mechanisms, possibly even acting in parallel, may underlie these associations. However, it should be noted that a shared postnatal stressful environment or, since this is not a genetically informed study, shared genes (Beijers et al., 2014) may also explain (part of) these findings.

3.4.3 Strengths and Limitations

Our longitudinal design allowed us to study the development of the cortisol circadian rhythm over a relatively long period in early childhood. The fact that we measured both prenatal, early postnatal (first 6 months) and later postnatal maternal distress (after the first 6 months), provides us with a full picture of the different associations during these pre- and postnatal periods of the child's life.

This study also has limitations, such as no assessments of maternal daily hassles at 30 and 72 months. Additionally, information regarding cortisol sampling (e.g., awakening and sampling times) was reported by the mothers, and preceding events (e.g., daily

activities, napping or feeding) were not taken into account, leaving room for potential compliance and confounding factors. Future research should control for these factors by using diaries and/or electronic sampling devices. Moreover, mothers were generally highly educated, limiting the generalizability of our results. Finally, it should be noted that the results are correlational, impeding causal conclusions.

3.4.4 Future Research

Earlier studies suggest that abnormal patterns in children's circadian cortisol might impact the development of the behavioral and emotional regulatory systems, subsequently leading to behavioral problems (Loman & Gunnar, 2010; Shirtcliff & Essex, 2008). Whether the alterations in the cortisol circadian rhythm found in the current study are also related to future behavioral and emotional problems remains to be determined.

Another open question is how the development of the cortisol circadian rhythm is associated with stress reactivity. Although humans normally respond to a stressful situation with an increase in cortisol (Dickerson & Kemeny, 2004), this response decreases during the first year of life and remains low throughout childhood (Lupien, McEwen, Gunnar, & Heim, 2009). Our results suggest that this may be accompanied by a general reduction in cortisol production. Future investigations of this potential overall reduction in HPA-axis activity in childhood, as well as of the link between the (development of) the cortisol circadian rhythm and stress reactivity are needed to further our knowledge of the mechanisms underlying HPA-axis development.

More research is also needed on the mechanisms underlying the links between maternal stress and anxiety and child circadian cortisol. Finally, we examined the independent associations between prenatal and postnatal maternal variables and child outcomes. Maternal postnatal distress might add on or amplify the effects of maternal prenatal distress on offspring. Large studies are needed to investigate how different combinations of prenatal and postnatal maternal stress and anxiety are linked to the development of the circadian rhythm in children.

3.4.5 Conclusions

Our study suggests that the cortisol circadian rhythm continues to develop beyond the first year of life and is characterized by a decrease of total cortisol concentrations between 1 and 6 years of age. Furthermore, our results suggest that stress early in the child's life, in the form of prenatal stress (maternal pregnancy-specific hassles) and postnatal maternal stress and anxiety, is associated with children's cortisol circadian rhythm between 1 and 6 years of age. Whether these associations are related to the development of (mental) health and behavior problems later in life remains to be determined.

Chapter 4

Child Stress Responses at Age 6 in the Light of Stress Early in Life

Based on:
Simons, S. S. H., Zijlmans, M. A. C., Cillessen A. H. N., & de Weerth, C. Child stress responses at age 6 in the light of stress early in life. Manuscript submitted for publication.

Abstract

Individuals differ in their physiological and behavioral stress responses and alterations in these responses have been associated with (mental) health. Therefore, it is important to understand the development and correlates of such stress responses. This study investigated potential predictors of physiological and behavioral stress responses of 6-year-old children to a stressful social evaluative situation (performance in front of a judge). Specifically, we investigated whether physiological (cortisol) and behavioral (gazing) stress responses were associated with environmental stress early in the child's life, in the form of maternal prenatal (late pregnancy, week 37) and early postnatal (first 6 months) distress. In addition, associations between the two stress responses were studied. To this end, 149 children (M_{age} = 6.09; 70 girls) participated in a social evaluative stress test (Children's Reactions to Evaluation Stress Test, CREST; de Weerth, Zijlmans, Mack, & Beijers, 2013a) in front of a judge. To operationalize physiological stress responses six cortisol saliva samples were collected and cortisol stress reactivity and total stress cortisol scores were calculated. To operationalize behavioral stress responses, gazing at the judge during the stress test was observed. Maternal prenatal distress was measured with questionnaires and physiological measures (cortisol saliva samples). Early postnatal distress was measured using questionnaires. Results indicated that less maternal fear of giving birth, higher maternal prenatal evening cortisol concentrations, and more maternal feelings of anxiety in the first 6 postnatal months all uniquely predicted higher total stress cortisol concentrations in children at age 6. Additionally, children with higher cortisol stress reactivity gazed less in the direction of the judge. These results suggest that maternal distress early in the child's life may program children's later HPA-axis functioning and that in 6-year-olds confronted with a stressful social situation, gazing may be used to deal with stress.

Keywords: child stress responses, gazing behavior, cortisol, maternal stress and anxiety, prenatal, early postnatal

4.1 Introduction

In stressful and/or threatening social situations individuals react physiologically and behaviorally. One physiological system that becomes activated is the hypothalamic-pituitary-adrenal (HPA) axis, producing its primary hormonal end-product cortisol, which mobilizes energy to respond (e.g., Nicolson, 2007). Behaviorally individuals may react, for example, by adjusting their attention (Wilson & MacLeod, 2003) and gazing at a threatening stimulus. Efficient stress responses are needed to deal with day-to-day stressors. However, there are individual differences in physiological and behavioral responses (e.g., Wilson & MacLeod, 2003) and alterations in these responses have been associated with (mental) health (e.g., Wilson & MacLeod, 2003; McEwen, 2008). Therefore, it is important to understand the development and correlates of such stress responses. The present study investigated the early life environment as a potential predictor of later stress responses. Both the uterine and early postnatal environments are thought to play an important role in shaping the development of the offspring's stress system (e.g., Seckl & Meaney, 2004; Loman & Gunnar, 2010). These effects may potentially affect development over a longer period of time or even permanently, and are called programming effects (e.g., Seckl & Meaney, 2004). In the current paper, we specifically studied associations between stress early in the child's life, in the form of maternal prenatal and early postnatal distress (i.e., stress and anxiety), and children's physiological (cortisol) and behavioral (gazing) responses to a social evaluative stressor at age 6.

Previous studies on the associations between maternal prenatal distress and child cortisol stress responses have rendered complex results. For example, in one study, more prenatal reported distress was associated with higher cortisol responses to a bathing session in 5-week-olds, and lower cortisol responses in 2- and 12-month-olds towards a vaccination and maternal separation, respectively (Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011). In another study, more perceived maternal prenatal stress was associated with higher cortisol reactivity to a heel-stick in newborns and to a toy removal task in 10-month-olds (Leung et al., 2010). Findings regarding the associations between maternal prenatal cortisol and child cortisol responses have also been mixed (for a review, see Zijlmans, Riksen-Walraven, & de Weerth, 2015a). For example, higher maternal prenatal cortisol was associated with larger cortisol responses to a heel stick in newborns and to a vaccination in 4- to 6-year-olds (Gutteling, de Weerth, & Buitelaar, 2004; Davis, Glynn, Waffarn, & Sandman, 2011), while Tollenaar et al. (2011) found no associations between maternal prenatal cortisol and child cortisol stress responses during children's first year.

Less is known about associations between maternal prenatal distress and children's gazing behavior in stressful and threatening situations. However, maternal distress may generally affect children's behavioral vigilance, and thereby their gazing behavior through prenatal programming. In line with this, infants of mothers who were more stressed during pregnancy looked away longer from the mother or objects used during a peek-a-boo task (Lin, Crnic, Luecken, & Gonzales, 2014). Prenatal distress has been associated with other child behavioral responses as well. For example, maternal prenatal life events were positively associated with observed fearfulness during a lab task in 14- to 19-month-olds (Bergman, Sarkar, O'Connor, Modi, & Glover, 2007). Further, associations have been found between anxiety and attention/gazing to threatening stimuli (Wilson & MacLeod, 2003), and between prenatal maternal anxiety and children's self-reported anxiety (8- and 9-year-olds; van den Bergh & Marcoen, 2004), suggesting that there may be associations between maternal prenatal distress and child gazing behavior in stressful and threatening situations.

Early maternal postnatal distress may also affect children's stress responses. Associations have been found between parenting stress and self-reported parenting, suggesting lower parenting quality, in parents of 2- to 5-year-olds (Anthony et al., 2005; Guajardo, Snyder, & Petersen, 2009). Lower parenting quality in turn may be stressful for young children (Loman & Gunnar, 2010). In line with this, mothers with an anxiety disorder were less sensitive and more intrusive, and had 9-month-olds with higher cortisol responses to a fearful situation than healthy controls (Feldman et al., 2009).

To our knowledge, research on associations between early postnatal maternal distress and child gazing behavior in stressful or threatening situations is lacking. Maternal distress early in children's lives may also affect their behavioral stress responses via caregiving. Braungart-Rieker, Garwood, Powers, and Notaro (1998) found that in 4-month-olds, mothers' sensitivity was positively associated with children's visual attention towards them during a stressful still face paradigm. Also 5- and 6-month-old children of responsive parents looked more at their parent during a procedure including free play, still faces, and reunions, than children of less responsive parents (Haley & Stansbury, 2003). Receiving lower quality care, which in itself may be considered a stressor, may affect the child's threat and stress response system as well as its behavior (Loman & Gunnar, 2010).

An additional goal of the present study was to investigate possible associations between cortisol responses and gazing behavior in response to the stressor. Previous studies on these types of associations are scarce and have yielded diverse findings. For example, in university students, larger increments of cortisol during a stressful interview were associated with more eye contact and engagement with the interviewers (Sgoifo et al., 2003). In contrast, in 6- to 17-year-olds, higher cortisol reactivity during a social

challenge (i.e., interview, singing, silent and oral reading) was associated with less gazing towards the interaction partner (Hessl, Glaser, Dyer-Friedman, & Reiss, 2006). Finally, in 10-year-olds no direct link between cortisol reactivity and gazing towards an evaluating judge was found (de Veld, Riksen-Walraven, & de Weerth, 2014).

In sum, the goals of the present study were to investigate whether maternal distress in late pregnancy and the first 6 postnatal months predicted 6-year-old's cortisol and gazing responses to a social evaluative stressor. Associations between children's cortisol responses and their gazing behavior during the stressor were also examined.

4.2 Methods

4.2.1 Participants

The data for this study were collected in an ongoing longitudinal project on the psychobiology of child development (BIBO project; Radboud University). Healthy mothers and their children are followed since late pregnancy. All mothers gave written informed consent; the study was approved by the Institutional Ethical Committee following the Helsinki Declaration (ECG 300107 and ECG 22111/130112). The project began with 220 mother-child dyads, 193 were still in the project when the child was 3 months old (see for details, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011a). At child age 6, 188 mother-child dyads were still in the project and were invited to participate in the 6-year data collection. Of this group, 149 children participated in a school visit that included a social evaluative stress test for which parents gave written informed consent (M_{age} = 6.09; *SD* = 0.14; *Min* = 5.87, *Max* = 6.85; 70 girls). Reasons for non-participation were: a preference of the school or child not to participate (*n* = 4), relocation of the family abroad (*n* = 3), or other reasons (e.g., parents saw the procedure as too challenging for their child, considered the project as too demanding, or had personal motives, *n* = 32). Of the invited 188 children, the 39 who did not participate did not differ significantly from the participating 149 children in maternal age at delivery, maternal educational level during pregnancy, child gender, or temperament (Children's Behavior Questionnaire short form; Putnam & Rothbart, 2006), all *p's* > .05. The current study used data collected prenatally (late pregnancy) and when the children were 3 months, 6 months, and 6 years old.

4.2.2 Procedure

4.2.2.1 Maternal prenatal distress

In late pregnancy, around the 37th week of pregnancy, mothers were asked to complete questionnaires regarding their feelings of general and pregnancy-specific stress and anx-

iety. They also were asked to collect diurnal saliva samples to determine their cortisol circadian rhythm.

4.2.2.2 Maternal early postnatal distress

When their child was 3 and 6 months old, mothers were asked to complete questionnaires about their feelings of general stress and anxiety.

4.2.2.3 Child stress responses at age 6

When children were 6 years old families were invited to let their child participate in a social evaluative stress test during a school visit. If parents agreed, the researchers visited the child's school with a mobile laboratory. In 8 of the 149 cases, the researchers visited the child at home with the mobile lab. All visits took place in the afternoon of a regular school day and started between 12:15 and 15:15 h. The visits began with the Children's Reactions to Evaluation Stress Test (CREST; de Weerth et al., 2013a; Simons, Cillessen, & de Weerth, 2017a, b) during which the child carried out three forced-failure tasks containing elements of unpredictability and uncontrollability in front of a judge. In the first task (restrained movement task; 1 min) children were asked to stand as still as possible, with an alarm clicked on their clothes. They were told that the alarm would beep if they moved. During the task the alarm went off twice on preprogrammed times independently of children's actual movement. In the second task (animal story task; 3 min) children listened to a recorded story about animals and were asked to fill in eight gaps in the story by making the sound of the animal just mentioned in the story. In this task children were told they would receive visual feedback from the judge (a green card) if they performed the sound perfectly. However, the judge showed the green card in only three of the eight sounds, irrespective of the child's actual performance. In the third task (tower of cans task; 3 min) children were asked to build a tower of horizontally lying cans which was almost impossible to do, but was told to be considered easy by peers (for details, see de Weerth et al., 2013a). After the three tasks, the judge left the room for 5 min to "evaluate" the child's performance. The total stress test procedure took 20 min (15 min for the three tasks, 5 min anticipation of the judge's evaluation). The CREST has been shown to effectively trigger an increase in cortisol concentrations in 5- and 6-year-olds (in an independent sample: de Weerth et al., 2013a; in this sample: Simons et al., 2017a, b). After the stress test, the judge told the child that he/she had performed perfectly well, the child was rewarded with a present, and a thorough debriefing took place. This was followed by a 25-minute recovery phase and another 25 min of tasks not described in this paper. The entire procedure was guided by a trained researcher who helped with saliva sampling, other tasks, and supported the child when necessary during the CREST.

4.2.3 Measures

4.2.3.1 Maternal prenatal and early postnatal distress

4.2.3.1.1 Pregnancy-specific anxiety

Two subscales of the Pregnancy-specific Anxiety Questionnaire-Revised (PRAQ-R; Buitelaar, Huizink, Mulder, Robles de Medina, & Visser, 2003; Huizink, Robles de Medina, Mulder, Visser, & Buitelaar, 2003; Huizink, Mulder, & Buitelaar, 2004a; Huizink, Mulder, Robles de Medina, Visser, & Buitelaar, 2004b) were used to measure pregnancy-specifix anxiety. The subscales measured fear of giving birth (3 items) and fear of bearing a handicapped child (4 items) using a 5-point scale. Cronbach's α was 0.73 and 0.82, respectively. Higher scores represented higher levels of maternal pregnancy-specific anxiety.

4.2.3.1.2 Pregnancy-specific stress

The Pregnancy Experience Scale (PES; DiPietro, Ghera, Costigan, & Hawkins, 2004) was used to measure pregnancy-specific stress. Each of 43-items in this questionnaire described a pregnancy-specific experience. Mothers rated on a 4-point scale to what degree each item resulted in a positive and a negative experience. Cronbach's α was 0.87 for positive ratings and 0.87 for negative ratings. To derive a score for the experienced negative emotional valence towards pregnancy, the sum of the negative ratings was divided by the sum of the positive ratings. Hence, higher scores represented a more negative emotional valence towards pregnancy that is, more pregnancy-specific daily hassles or stress.

4.2.3.1.3 General anxiety

Prenatally, and when children were 3 and 6 months old, maternal feelings of general anxiety were assessed using the state subscale of the State-Trait Anxiety Inventory (STAI; van der Ploeg, Defares, & Spielberger, 1981; Spielberger, 1983). This scale consists of 20-items answered on a 4-point scale. Higher scores represented higher levels of maternal state anxiety. Cronbach's α ranged from 0.90 to 0.93. To operationalize maternal postnatal anxiety in the first 6 postnatal months the average of the 3 and 6 month scores (Spearman's *rho* = .53, $p < .01$) was computed. This questionnaire was also used when the child was 6 years old (see Section 4.2.3.3).

4.2.3.1.4 General stress

Prenatally, and when children were 3 and 6 months old a Dutch questionnaire (Alledaagse Problemen Lijst; APL; Vingerhoets, Jeninga, & Menges, 1989; test-retest reliabilities 0.76-0.87) was used to measure maternal stress. This questionnaire has 49 items describing daily hassles. Mothers indicated whether each item had occurred in the last two months, and, if so, rated how much it had bothered them using a 4-point scale. To derive a mean intensity rating the sum of the ratings given was divided by the number of occurred

events. Higher scores thereby represented higher levels of experienced negativity due to daily hassles, indicating more stress. Early maternal postnatal stress (first 6 months) was operationalized as the average of the 3 and 6 month scores (Spearman's *rho* = .57, $p < .01$).

4.2.3.1.5 Cortisol

During pregnancy, mothers were asked to collect diurnal saliva samples on two consecutive days at awakening (T1), 30 min after awakening (T2), at 12:00 h (T3), 16:00 h (T4), and 21:00 h (T5). Samples were collected by passive drooling and stored in a freezer (-25 °C). Subsequent cortisol analyses were carried out at the Laboratory of Endocrinology of the University Medical Center Utrecht (see, for details, Simons, Beijers, Cillessen, & de Weerth, 2015; Simons, Cillessen, & de Weerth 2017a). Fluctuations in cortisol concentrations were diminished by excluding samples (5.7%) that deviated too much from the required sampling times as well as samples collected during or after the day of delivery (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2010). Mean concentrations over the two days were calculated (Beijers et al., 2010). To operationalize maternal physiological distress, diurnal cortisol decline (T1 minus T5) and evening cortisol (T5) were calculated. These measures were consistent with earlier research (Beijers et al., 2010) in which they were found to correlate highly with other prenatal maternal cortisol measures (total amount of diurnal cortisol, morning cortisol concentrations, and cortisol awakening response). Higher scores on diurnal cortisol decline represented a steeper diurnal decline in cortisol concentrations.

4.2.3.2 Child stress responses

4.2.3.2.1 Physiological stress responses: cortisol responses

At child age 6, six saliva samples (C1-C6) were collected from the child during the stress test procedure to determine baseline, response, and recovery cortisol concentrations. As in the original CREST, baseline cortisol concentrations (C1 and C2) were collected immediately before the CREST started (C1) and 15 min after test onset (C2; de Weerth et al., 2013a; Simons et al., 2017a). Because physical activity and eating can affect cortisol concentrations (e.g., Dickerson & Kemeny, 2004; Nicolson, 2007), children were asked not to eat, drink, or be physically active 30 min prior to the test. Samples representing cortisol concentrations in response to the stressor (C3 and C4) were obtained 25 and 35 min after test onset. Samples representing cortisol concentrations during a recovery period (C5 and C6) were obtained 50 and 58 min after test onset. All saliva samples were collected using eye sponges (BD Visispeare, Waltham, MA; de Weerth, Jansen, Vos, Maitimu, & Lentjes, 2007). Centrifuged samples were stored at -25 °C and analyzed in the Laboratory of Endocrinology of the University Medical Center Utrecht (see, for details, Simons at al., 2015; 2017a).

Cortisol concentrations of 4 of the 149 children were excluded from all analyses because the children used medication that could affect their cortisol concentrations (*n* = 3) or because the timing of the saliva samples differed largely from the standard sampling times in the protocol (*n* = 1). A paired samples *t*-test indicated that the stress test induced a significant increase in cortisol from the lowest baseline concentrations (*M* = 6.06 nmol/L, *SD* = 2.70) to the highest peak response concentrations (*M* = 7.12 nmol/L, *SD* = 3.79), $t(141) = -4.41$, $p < .01$ (see Simons et al., 2017a (Chapter 5), for a figure representing mean scores and standard errors for each of the six sampling moments). Two indices of the cortisol stress response were calculated. Total stress cortisol was calculated as the area under the curve across all six samples: AUC = (C2 + C1) x 15/2 + (C3 + C2) x 10/2 + (C4 + C3) x 10/2 + (C5 + C4) x 15/2 + (C6 + C5) x 8/2. Cortisol stress reactivity was calculated by saving the standardized residual scores of a regression predicting the highest peak response concentrations from the lowest baseline concentrations (Schuetze, Lopez, Granger, & Eiden, 2008; de Veld, Riksen-Walraven, & de Weerth, 2012; Simons et al., 2017a). Total stress cortisol and cortisol stress reactivity scores were log transformed to increase the normal distribution of the residuals of the regression analyses.

4.2.3.2.2 Behavioral stress responses: gazing behavior

Gazing behavior was determined during the second subtest of the stress test, the animal story task. This subtest was chosen for the gazing observations because it is most similar to the Trier Social Stress Test (Kirschbaum, Pirke, & Hellhammer, 1993), in that the child could move freely (as opposed to the restrained movement task), and was not focused on a manual task (as opposed to the tower of cans task). To observe gazing behavior during this test children were videotaped while performing, using a camera positioned above the judge. The videotapes were coded using Noldus Observer XT (Observer XT, version 11) and an ethogram developed to observe behavior during the CREST. Two independent observers coded the video's using interval coding, scoring the child's gaze direction every two seconds (see Table 4.1 for the ethogram of gazing behavior). Categories of the gazing behavior ethogram were mutually exclusive and hierarchically ordered. Scores for gazing behavior were calculated by counting the number of intervals in which the child looked at the judge (sum of "looks at judge and experimenter" and "looks at judge") divided by the total number of intervals (sum of all categories minus intervals in which gazing behavior could not be scored: "not visible"). Of the 142 videos, 7 could not be scored due to technical problems at recording. Of the 135 videos that were coded, inter-rater reliability between the two observers was calculated over 10 randomly selected videos and was excellent, Cohen's $k = 0.83$. Higher scores on gazing behavior indicated more gazing towards the judge.

Table 4.1 | *Ethogram for Gazing Behavior During the Stress Test*

Gazing behavior	Definition
Looks at judge and experimenter	Child looks at judge and experimenter at any moment within two second interval
Looks at experimenter	Child looks at experimenter at any moment within two second interval
Looks at judge	Child looks at judge at any moment within two second interval
Looks elsewhere	Child does not look at judge or experimenter at any moment during the two second interval, but looks upwards, downwards, or to the side
Eyes closed	Child has his/her eyes closed during whole two second interval
Not visible	Eyes are not visible during whole two second interval

Note. Proportion scores were corrected for the intervals that were "not visible".
For the statistical analyses, categories "looks at judge and experimenter" and "looks at judge" were added up to represent the total number of intervals in which the child looked at the judge.

4.2.3.3 Potential confounders

Child gender (*girl* = 0, *boy* = 1), maternal educational level at child age 6, and maternal anxiety at child age 6 were considered potential confounders. Maternal educational level was mothers' highest educational level ranging from "*primary*" (1) to "*university*" (8), followed by the option "*other*". "Other" answers (n = 5) were recoded into the closest matching option. Maternal anxiety at child age 6 was derived from the state subscale of the State-Trait Anxiety Inventory (see Section 4.2.3.1.3). Cronbach's α was 0.93 for this measurement at child age 6.

4.2.4 Statistical Analyses

To investigate associations between maternal distress (prenatal and during the first 6 postnatal months) and child stress responses, three hierarchical regressions were run with total stress cortisol, cortisol stress reactivity, and gazing behavior as dependent variables. In each regression, confounders that were significantly correlated with the outcome variable were included first, in Step 1 (Tabachnick & Fidell, 2007). Subsequently, the prenatal and early postnatal measures of maternal distress were added. To investigate how physiological and behavioral stress responses were associated, Spearman correlations were calculated.

4.3 Results

Descriptive statistics of the untransformed study variables are presented in Table 4.2. Spearman correlations are presented in Table 4.3. Higher cortisol stress reactivity scores were associated with less gazing at the judge (Spearman's *rho* = -.17, $p < .05$). Higher levels of maternal prenatal evening cortisol were associated with higher total stress cortisol concentrations (Spearman's *rho* = .25, $p < .01$) and higher cortisol stress reactivity scores at age 6 (Spearman's *rho* = .25, $p < .01$). Because girls' gazing was more directed towards the judge than boys' gazing (Spearman's *rho* = -.21, $p = .01$), child gender was included as a confounder in the regression predicting gazing behavior. Because maternal anxiety at age 6 was associated with higher total stress cortisol concentrations at age 6 (Spearman's *rho* = .17, $p < .05$), maternal anxiety at age 6 was included as a confounder in the regression predicting children's total stress cortisol concentrations.

Table 4.2 | *Descriptive Statistics of all Study Variables*

	N	*M*	*SD*	*Min*	*Max*
Confounders					
Child gender (% girls)	149	47.0%			
Maternal educational level	144	6.76	1.39	3.00	8.00
Maternal anxiety, age 6	143	30.95	8.43	20.00	68.00
Predictors					
Maternal Prenatal					
Daily hassles	136	1.14	0.44	0.00	2.54
Anxiety	136	32.02	8.93	20.00	64.00
Pregnancy-specific hassles	136	0.32	0.22	0.00	1.43
Fear of giving birth	136	5.45	2.58	3.00	15.00
Fear of bearing a handicapped child	136	9.13	3.37	4.00	20.00
Diurnal cortisol decline (nmol/L)	119	6.75	4.50	-2.80	24.00
Evening cortisol (nmol/L)	125	9.72	3.73	0.85	37.00
Maternal Postnatal					
Anxiety, mean 3 and 6 months	149	28.70	6.64	19.50	59.50
Daily hassles, mean 3 and 6 months	149	1.15	0.38	0.00	2.28
Outcomes (child)					
Total stress cortisol (AUC)	134	375.80	169.48	73.80	1474.50
Cortisol stress reactivity[a]	142	0.00	1.00	-1.77	5.15
Gazing behavior	142	0.28	0.12	0.01	0.50

Note. AUC = area under the curve (total stress cortisol concentration).

[a]Due to the operationalization of reactivity as standardized residuals the mean of this variable is 0.00 and the *SD* is 1.00.

Table 4.3 | *Spearman Correlations Between all Study Variables*

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13[a].	14[a].
Confounders														
1. Child gender														
2. Maternal educational level	-.02													
3. Maternal anxiety, age 6	.13	-.01												
Predictors														
Maternal prenatal														
4. Daily hassles	-.20*	-.03	.11											
5. Anxiety	.08	.03	.46**	.23**										
6. Pregnancy-specific hassles	-.09	.01	.34**	.22*	.40**									
7. Fear of giving birth	-.11	.09	.25**	.08	.40**	.32**								
8. Fear of bearing a handicapped child	-.10	-.04	.07	.13	.11	.20*	.19*							
9. Diurnal cortisol decline (nmol/L)	-.02	.02	-.01	-.09	-.10	.16[+]	-.09	-.19*						

Table 4.3 | *(continued)*

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13[a].	14[a].
10. Evening cortisol (nmol/L)	-.05	-.01	.13	-.04	.03	-.20*	.05	-.02	-.24**					
Maternal postnatal														
11. Anxiety, mean 3 and 6 months	-.01	.11	.61**	.17*	.55**	.41**	.39**	.16^{+}	-.03	.01				
12. Daily hassles, mean 3 and 6 months	-.15^{+}	.04	.25**	.46**	.23**	.41**	.17*	.15^{+}	.03	-.10	.36**			
Outcomes (child)														
13.Total stress cortisol (AUC)[a]	-.04	-.05	.17*	-.08	.02	.04	-.10	-.13	.01	.25**	.11	.03		
14. Cortisol stress reactivity[a]	.10	-.12	-.03	.07	-.12	-.09	-.10	-.15^{+}	-.15	.25**	-.06	-.12	.46**	
15. Gazing behavior	-.21**	.02	-.01	-.08	-.13	-.02	-.09	.02	-.01	-.03	-.00	-.01	-.14	-.17*

Note. AUC = area under the curve (total stress cortisol concentration).

[a]log transformed.

$^{+}p \leq .10$, * $p \leq .05$, ** $p \leq .01$.

Due to missing data, 30 of the 149 children were dropped out of all three regression analyses. These 30 children did not differ significantly from the other 119 children in gender, temperament, or maternal age or educational level, all *p's* > .05.

In the regression predicting total stress cortisol, Step 1 was not significant, $p > .05$ (see Table 4.4). Step 2 had significant additive value, $F_{change}(9, 95) = 2.04$, $p < .05$, $R^2_{change} = .16$, and the total model at Step 2 also was significant, $F(10, 95) = 1.92$, $p = .05$, $R^2 = .17$ (see Table 4.4). Maternal fear of giving birth, maternal prenatal evening cortisol concentrations, and maternal feelings of anxiety in the first 6 postnatal months of the child's life were all uniquely associated with children's total stress cortisol concentrations (all *p's* < .05; see Table 4.4). Higher maternal fear of giving birth was associated with lower total stress cortisol at child age 6. Higher prenatal maternal evening cortisol concentrations and higher maternal feelings of anxiety in the first 6 postnatal months were associated with higher total stress cortisol concentrations at age 6 (see Table 4.4).

The regression predicting cortisol stress reactivity was not significant, $p > .05$ (see Table 4.4).

In the regression predicting gazing behavior, Step 1 was significant, $F(1, 114) = 5.84$, $p < .05$, $R^2 = .05$: girls looked more in the direction of the judge than boys (see Table 4.4). Step 2 did not have significant additive value and the model at Step 2 was not significant, both *p's* > .05 (see Table 4.4).

4.4 Discussion

In this study, the correlates of physiological and behavioral stress responses to a social evaluative stress paradigm were investigated in 6-year-old children. Specifically, we examined whether physiological stress responses (total stress cortisol and cortisol stress reactivity) and behavioral stress responses (gazing) were predicted by stress early in the child's life, as indicated by maternal prenatal and early postnatal distress. The associations between the two stress responses (physiological and behavioral) were also examined. Results indicated that less maternal fear of giving birth, higher maternal prenatal evening cortisol, and more maternal early postnatal anxiety were all uniquely predictive of higher child total stress cortisol concentrations at age 6. Additionally, higher cortisol stress reactivity was associated with less gazing at the judge during the social evaluative stress test.

Table 4.4 | *Results from Regressions Predicting Total Stress Cortisol, Cortisol Stress Reactivity, and Gazing Behavior from Prenatal and Early Postnatal Maternal Distress*

	Model 1		Model 2	
	B	*ß*	*B*	*ß*
Total Stress Cortisol[a]				
Step 1				
Anxiety, 6 years	< 0.01	.08	< -0.01	-.18
Step 2				
Daily hassles, prenatal			0.01	.02
Anxiety, prenatal			< -0.01	-.03
Pregnancy-specific hassles, prenatal			0.06	.07
Fear of giving birth, prenatal			-0.02	-.26*
Fear of bearing a handicapped child, prenatal			< -0.01	-.07
Diurnal cortisol decline (nmol/L), prenatal			< 0.01	.01
Evening cortisol (nmol/L), prenatal			0.01	.23*
Anxiety, mean 3 and 6 months			0.01	.44**
Daily hassles, mean 3 and 6 months			0.02	.05
R^2_{change}	.01		.16*	
R^2_{model}	.01		.17*	
Cortisol Stress Reactivity[a]				
Daily hassles, prenatal	0.09	.21[+]		
Anxiety, prenatal	< -0.01	-.07		
Pregnancy-specific hassles, prenatal	0.04	.04		
Fear of giving birth, prenatal	-0.02	-.23*		
Fear of bearing a handicapped child, prenatal	< -0.01	-.08		
Diurnal cortisol decline (nmol/L), prenatal	< -0.01	-.07		
Evening cortisol (nmol/L), prenatal	0.01	.17[+]		
Anxiety, mean 3 and 6 months	< 0.01	.16		
Daily hassles, mean 3 and 6 months	-0.07	-.17		
R^2_{change}	.12			
R^2_{model}	.12			

Note. No outliers were removed because Cook's distances indicated no potentially influential data points.
[a]log transformed.
[+] $p \leq .10$, * $p \leq .05$, ** $p \leq .01$.

Table 4.4 | *Results from Regressions Predicting Total Stress Cortisol, Cortisol Stress Reactivity, and Gazing Behavior from Prenatal and Early Postnatal Maternal Distress (continued)*

	Model 1		Model 2	
	B	*ß*	*B*	*ß*
Gazing Behavior				
Step 1				
Child gender	-0.05	-.22*	-0.05	-.21*
Step 2				
Daily hassles, prenatal			< -0.01	< -.01
Anxiety, prenatal			< -0.01	-.22^{+}
Pregnancy-specific hassles, prenatal			0.04	.06
Fear of giving birth, prenatal			< 0.01	.03
Fear of bearing a handicapped child, prenatal			< 0.01	< .01
Diurnal cortisol decline (nmol/L), prenatal			< 0.01	< .01
Evening cortisol (nmol/L), prenatal			< 0.01	.01
Anxiety, mean 3 and 6 months			< 0.01	.16
Daily hassles, mean 3 and 6 months			-0.01	-.05
R^2_{change}	.05*		.04	
R^2_{model}	.05*		.09	

Note. No outliers were removed because Cook's distances indicated no potentially influential data points.
$^{+}p \leq .10$, * $p \leq .05$, ** $p \leq .01$.

4.4.1 Stress Early in Life and Child Stress Responses

The finding that prenatal maternal evening cortisol, fear of giving birth, and maternal early postnatal (first 6 months) anxiety were associated with total stress cortisol of 6-year-olds may suggest that prenatal and early postnatal maternal distress affect the development of the offspring's HPA-axis. This is in line with programming hypotheses and the idea that stress early in life is associated with later HPA-axis functioning (e.g., Seckl & Meaney, 2004; Loman & Gunnar, 2010; Chaby, 2016).

The positive association between maternal evening cortisol and child total stress cortisol may be explained by a programming effect of higher maternal cortisol concentrations on children's HPA-axis development. In rats, maternal prenatal stress is linked to the offspring's number of hippocampal corticosteroid receptors and diurnal activity of the HPA-axis (Koehl et al., 1999). In humans, maternal cortisol may affect the fetal developing brain and HPA-axis in various manners. For example, maternal cortisol may

stimulate placental production of corticotrophin releasing hormone (CRH), which in turn may stimulate fetal production of (excessive) cortisol, affecting the developing fetus (Zijlmans et al., 2015a). It is also possible that maternal cortisol reduces the placental blood flow which in turn may stress the fetus, thereby affecting the developing HPA-axis (e.g., Huizink et al., 2004a; Beijers, Buitelaar, & de Weerth, 2014; Zijlmans et al., 2015a). Alternatively, high maternal evening cortisol concentrations may affect the fetus more indirectly, through maternal behaviour. For example, maternal sleep may be disturbed, in turn affecting maternal metabolic and inflammatory systems (see Beijers et al., 2014), thereby potentially affecting fetal development.

The positive association between maternal anxiety in the first 6 postnatal months and total stress cortisol of 6-year-olds may be explained by lower quality caregiving. This is in line with research indicating that mothers with an anxiety disorder are less sensitive and more intrusive and have 9-month-olds with higher cortisol stress responses (Feldman et al., 2009). An anxious mother may herself be a source of stress for the child if her anxiety leads to lower quality care (Loman & Gunnar, 2010), or an anxious mother may be less able to buffer her child from environmental stress. In both cases, children's HPA-axis functioning may be affected. Alternatively, heightened stressfulness in the shared mother-child environment may (partly) explain this association.

The finding that children of mothers with more fear of giving birth had lower total cortisol concentrations seems contradictory to our other findings described above, and is difficult to explain. Not much is known about the effects of specific types of prenatal or pregnancy-related anxieties on women's physiological states and behavior. It could be hypothesized that fear of giving birth is associated with higher cortisol in the mother. These increased cortisol concentrations would then affect fetal HPA-axis development. However, in our sample the association between maternal evening cortisol and child total stress cortisol was positive, whereas the association between maternal fear of giving birth and child total stress cortisol was negative. There was also no evidence found for associations between maternal fear of giving birth and maternal evening cortisol or diurnal cortisol decline. This could be suggesting that mechanisms other than cortisol may underlie the association between fear of giving birth and child total cortisol concentrations. For example, fear of giving birth could affect maternal lifestyle, such as eating or exercise (Beijers et al., 2014), in turn affecting the child's prenatal environmental conditions and potentially HPA-axis development. However, how this would result in lower instead of (as found for maternal prenatal evening cortisol and maternal anxiety during the first 6 postnatal months) higher total stress cortisol in the child 6 years later remains unknown. Moreover, other research on the associations between maternal fear of giving birth and children's HPA-axis functioning did not find support for associations between

both factors (Gutteling et al., 2004; Tollenaar et al., 2011; Simons et al., 2015). Future in-depth research on maternal prenatal and pregnancy-related stress and anxiety, lifestyle, and physiology is needed to shed more light on this subject.

The regressions did not support associations between prenatal and early postnatal maternal distress and gazing or cortisol stress reactivity. These results may suggest that 6-year-old children's gazing and cortisol reactivity during stress are independent of maternal prenatal and early postnatal distress. However, study characteristics may also explain these findings. For example, in our stress test protocol the judge provided the children with visual feedback on their performance (showed a green card); this may have constrained spontaneous gazing behavior and affected the stressfulness of the situation. Also, another type of stressor (e.g., a physical stressor) may have produced different patterns of reactions in the child as well as different links with stress early in the child's life. Additionally, although previous research suggests that the (early life) environment is linked to later HPA-axis functioning (e.g., Loman & Gunnar, 2010), these links may be particularly salient in children experiencing more severe environmental stress than the children in our middle class sample.

The fact that regression analyses provided support for the idea that stress early in life predicted children's total cortisol during the stress protocol, but not for a link with their cortisol stress reactivity, may also be related to the nature of these measures. While cortisol reactivity reflects the dynamics of the response to the stressor, total stress cortisol reflects cortisol concentrations during the entire procedure. As such, this last measure may more generally represent cortisol production during a school day or any day in general (circadian cortisol) than in reaction to the stressor per se. The results may be suggesting that stress early in life is more predictive of basal functioning than physiological (and even behavioral) reactivity in response to a specific acute stressor. In line with this are the earlier links found by Simons et al. (2015) between stress early in life and markers of cortisol circadian rhythm in early childhood, and the positive association between total circadian and total stress cortisol (Simons et al., 2017b). Acute stress responses may be more variable, depending on the specific situation and stressor, and hence more difficult to link to stress early in life.

4.4.2 Associations Between Physiological and Behavioral Stress Responses

The negative association between gazing at the judge and cortisol reactivity scores is in line with a negative association between cortisol reactivity and gazing towards an interaction partner during a social challenge in 6- to 17-year-olds (Hessl et al., 2006). However, it is not in line with associations between larger increments of cortisol and more eye contact in adolescents during a social challenge (Sgoifo et al., 2003) and research in

10-year-olds showing no direct link between gaze aversion and cortisol reactivity during a social challenge (de Veld et al., 2014). The differences may be explained by differences in the stressors that were used. Sgoifo et al. (2003) used an interview in which students talked about their distinctive personality features, not resulting in a significant increase in cortisol in the group as a whole. De Veld et al. (2014) used a paradigm comparable to ours (performance in front of a judge), that lead to significant increases in cortisol in the group as a whole. Our protocol triggered looking at the judge for feedback; this was not the case in de Veld et al. (2014) where (verbal) feedback was provided only if mistakes were made or the child fell silent. These features may have affected gazing and/or cortisol responses, as well as their associations.

The negative association between gazing and cortisol stress reactivity scores in our study may suggest that keeping an eye on the judge is associated with lower cortisol stress reactivity. Children may have used gazing towards the information-providing judge in combination with cognitive strategies to reduce physiological arousal. This combination may have increased feelings of control, hence reducing uncontrollability and physiological responses (Dickerson & Kemeny, 2004). In line with this it has been found that between 6 and 10 years children start to shift from predominately behavioral strategies (such as gaze aversion) to more cognitive emotion regulation strategies (Terwogt & Stegge, 1995). However, since false feedback was provided, uncontrollability probably kept playing a role. And given the correlational design, directions may be reversed: experiencing the situation physiologically as less stressful may result in more (relaxed) gazing at the judge. Other factors, such as shyness, also may have played a role. More shyness may result in less looking at the judge and higher physiological responses. Indeed, shyness predicted higher cortisol reactivity in response to meeting a stranger in 3-year-olds (Zimmerman & Stansbury, 2004). To date, few studies of stress responses include measures of behavior. More observational research in combination with experimental designs is needed to elucidate if children use behavior to deal with physiological stress. Experimental studies of behavior under stress, for example by training children to look more or less at the judge, may clarify the potential regulatory functions of behavior which in turn may benefit future interventions.

That no association between gazing and total stress cortisol was found may be because gazing behavior, just as cortisol reactivity, reflects the dynamics of the responses to the stressor, while the total stress cortisol represents cortisol secretion during the entire protocol (i.e., including cortisol before and after the stressor).

4.4.3 Strengths and Limitations

Strengths of this study are the relatively large longitudinal sample, the combination of physiological, behavioral, and psychological measures, and the use of an effective stress

test for 6-year-olds. However, the study is limited in generalizability because the sample was primarily middle class. Additionally, the use of a specific (social evaluative) stressor limits generalizability to other stressful situations, and because the study was not genetically informed, genetic or epigenetic (Meaney, 2010) transference cannot be ruled out. Finally, the correlational design precludes causal conclusions.

4.4.4 Future Research

To increase generalizability, links between stress early in life and children's stress responses as well as between physiological and behavioral stress responses should be studied in various contexts and environments. Since gazing may modify physiological responses to acute stress it is interesting for future (intervention) research to study the causality of the links between physiological and behavioral stress responses. Moreover, gazing may moderate associations between stress early in life and children's HPA-axis functioning, possibly explaining the mixed findings of previous research. In this study, the sample size prevented testing this but future research with larger sample sizes could test moderation by behavioral strategies. Finally, since stress responses may continue to develop after age 6, it is interesting to examine how stress responses at older ages relate to stress early in life and what role cognitive development plays in these associations.

4.4.5 Conclusions

Maternal distress early in the child's life was associated with 6-year-old children's total cortisol secretion in a stressful social evaluative situation. Additionally, higher cortisol reactivity was associated with less gazing at the judge. The results suggest that environmental stress early in life may program children's later HPA-axis functioning and that 6-year-olds confronted with a stressful social situation may use gazing to deal with stress.

Chapter 5

Cortisol Stress Responses and Children's Behavioral Functioning at School

Based on:

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Abstract

The present study investigated whether cortisol stress responses of 6-year-olds were associated with their behavioral functioning at school. Additionally, the moderating role of stress in the family environment was examined. To this end, 149 healthy children (M_{age} = 6.09 years; 70 girls) participated in an age-appropriate innovative social evaluative stress test. Saliva cortisol samples were collected six times during the stress test to calculate two indices of the cortisol stress response: cortisol stress reactivity and total stress cortisol. Teachers assessed children's internalizing, externalizing, and prosocial behaviors. Stress in the family environment was operationalized as maternally reported parenting stress. Results indicated a significant increase in cortisol concentrations in response to the stressor. No significant associations were found between cortisol stress responses and behavioral functioning at school and there was no evidence for moderation by maternal parenting stress. Potential theoretical and methodological explanations for these results are discussed.

***Keywords**:* cortisol stress responses, family stress, behavior, teacher report, children

5.1 Introduction

Normally developing children differ in how they behave at school. Behavioral functioning at school influences a child's peer relations and learning, and in turn the child's future. This study aimed to obtain more insight in the correlates of behavioral functioning at school by focusing on the role of children's cortisol stress responses. Furthermore, the moderating role of stress in the family environment in the form of maternal parenting stress was studied.

In stressful situations the hypothalamic-pituitary-adrenal (HPA) axis, with its primary hormonal end product cortisol, becomes activated. The HPA-axis prepares the individual to respond behaviorally and physiologically to a stressor. There are individual differences in this normative HPA-axis response (Kudielka, Hellhammer, & Wüst, 2009). Moreover, both HPA-axis functioning and behavioral tendencies appear to be trait-like characteristics of a person (e.g., high vs. low reactive phenotype; Boyce & Ellis, 2005).

Links between HPA-axis functioning and trait-like behavior in different contexts have been described. For example, in 4.5-year-olds higher cortisol reactivity in an emotion eliciting task, was associated with more mother reported dispositional effortful control (Spinrad et al., 2009). In 4- to 5-year-olds, a moderate increase in cortisol followed by down regulation was associated with more self-regulation, while higher baseline cortisol and blunted cortisol reactivity/decreasing cortisol was associated with more aggression (Blair, Granger, & Razza, 2005). Higher cortisol reactivity was associated with less school engagement, less academic competence, and more externalizing behavior (boys only) in 5- to 6-year-olds (Obradović, Bush, Stamperdahl, Adler, & Boyce, 2010). In 11-year-olds, lower cortisol reactivity was associated with more externalizing behaviors and delinquency whereas higher cortisol reactivity was associated with positive student-teacher relationships (Conradt et al., 2014). Others found no association between cortisol reactivity and impulsivity, internalizing, and externalizing behavior (e.g., Alink et al., 2008; Spinrad et al., 2009). In sum, this indicates that to date findings regarding associations between child cortisol stress responses and trait-like behavioral functioning are varied and somewhat inconsistent.

These inconsistencies may be explained by moderators. One potential moderator may be stress in children's social environment. Quellet-Morin et al. (2011) showed that lower cortisol responses were associated with more behavioral and social problems, but only in bullied and/or maltreated children. Moreover, the interaction between family environment and cortisol reactivity predicted prosocial behavior. More family adversity was associated with less prosocial behavior but only in high cortisol reactive children (Obradović et al., 2010). Potentially, environmental stress "gets under the skin" not only

affecting the HPA-axis (e.g., Kudielka et al., 2009; Loman & Gunnar, 2010; Quellet-Morin et al., 2011) and/or behavioral functioning (e.g., Anthony et al., 2005; McCarty, Zimmerman, Digiuseppe, & Christakis, 2005; Pachter, Auinger, Palmer, & Weitzman, 2006) but also the associations between both.

A specific factor affecting stress in children's social environment may be stress in the family environment in the form of maternal parenting stress. Maternal parenting stress may affect the child's daily environment by shaping maternal behavior. Indeed, parenting stress is associated with lower self-reported parenting quality, as evidenced by, for example, more parental laxness, stricter discipline, less nurturing behavior, and lower expectations of the child (Anthony et al., 2005; Guajardo, Snyder, & Petersen, 2009). Moreover, in these studies parenting stress was also associated with more internalizing and externalizing behaviors, and less social competence in children.

The goal of the current study was to extend the above described knowledge by exploring associations between cortisol stress responses and behavioral functioning at school in a non-clinical sample of 6-year-old children. Additionally, the *moderating* role of stress in the family environment, operationalized as maternal feelings of parenting stress was investigated. The age of 6 was chosen because at this age, due to formalization of evaluation at school in many countries, as well as the use of impression management and the development of relief and regret, children may become more exposed and vulnerable to social evaluative stress (e.g., Dickerson & Kemeny, 2004; Engelmann, Herrmann, & Tomasello, 2012; Weisberg & Beck, 2012). To determine the cortisol stress response an innovative, effective (i.e., triggering a significant increase in cortisol), age-appropriate social evaluative stressor was used (de Weerth, Zijlmans, Mack, & Beijers, 2013a). Finally, two indices of cortisol stress responses were calculated: total stress cortisol (i.e., including baseline/anticipation, stressor, and recovery periods), and cortisol stress reactivity (i.e., change in response to the stressor).

5.2 Methods

5.2.1 Participants

This study was part of an ongoing longitudinal project that started during pregnancy and focuses on the psychobiological factors associated with children's development (BIBO project; Radboud University). The project and the current cross-sectional data collection were approved by the Institutional Ethical Committee, which follows the Helsinki Declaration (ECG 300107 and ECG 22111/130112, respectively). Participants in the total project were 220 healthy born children and their mothers of whom 193 were still in

the project 3 months postpartum (for details, see Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011a; Beijers, Riksen-Walraven, & de Weerth, 2013a; Beijers, Riksen-Walraven, Putnam, de Jong, & de Weerth, 2013b). Of this group, the 188 dyads who were still in the study around the child's 6th birthday were invited to participate in the current data collection. Of the invited group, 149 children participated in a school visit after their parents gave informed consent (M_{age} = 6.09; *SD* = 0.14; *Min* = 5.87, *Max* = 6.85; 70 girls). Reasons for non-participation were: child or school preferred not to participate (*n* = 4), family had moved abroad (*n* = 3), or parents declined to participate for other reasons (e.g., they considered the procedure too challenging for their child, they considered the study as too intensive, or personal reasons, *n* = 32). Children who did not participate (*n* = 39) did not differ significantly from the participating children in educational level of the mother during pregnancy, age of the mother at delivery, gender of the child, or child age four temperament (Children's Behavior Questionnaire short form; CBQ short form; Putnam & Rothbart, 2006), all *p's* > .050.

5.2.2 Procedure

5.2.2.1 School visit

Children were tested in the afternoon of a regular school day in a mobile laboratory (van) parked near their school (or home *n* = 8 of 149). After entering the mobile lab children participated in the Children's Reactions to Evaluation Stress Test (CREST), a social evaluative stress test during which the child carries out three forced-failure tasks containing elements of unpredictability and uncontrollability in front of a "judge" who evaluates their performance (for details, see de Weerth et al., 2013a). The procedure takes 20 min (task performance and anticipation of the judge's evaluation) and is stressful for 5- to 6-year-old children as indicated by increases in cortisol concentrations (de Weerth et al., 2013a). After the procedure thorough debriefing took place, in which the child was shown that the tasks were rigged and was told that he/she had performed well and therefore deserved a present. The child then was allowed to draw and watch movies during a 25-minute recovery phase, followed by 25 min of unrelated tasks (not described/used in this manuscript).

5.2.3 Measures

5.2.3.1 Cortisol

During the school visit, six saliva samples (C1-C6) were collected from each child. In line with the original CREST (de Weerth et al., 2013a) baseline cortisol concentrations were obtained directly before the CREST (children were asked not eat, drink, or be physically active 30 min prior to the school visit), and 15 min after the start of the CREST, C1 and

C2, respectively. To measure cortisol concentrations in response to the CREST, C3 and C4 were obtained 25 and 35 min after the start of the CREST, respectively. To measure recovery cortisol concentrations, C5 and C6 were obtained 50 and 58 min after the start of the CREST. In order to avoid interference of the cortisol circadian rhythm all test sessions started in the afternoon (Dickerson & Kemeny, 2004) between 12:15 and 15:15 h. If a child was ill on the planned testing day the school visit was rescheduled.

Saliva was collected using eye sponges (BD Visispeare, Waltham, MA; de Weerth, Jansen, Vos, Maitimu, & Lentjes, 2007) which participants had to put in their mouth for approximately 1 min. Saliva samples, obtained by centrifuging the eye sponges, were stored in a freezer (-25 °C). Subsequently, cortisol analyses were carried out at the Laboratory of Endocrinology of the University Medical Center Utrecht. An in-house competitive radio-immunoassay was used with a polyclonal anticortisol-antibody (K7348) and [1,2-^{3}H(N)]-Hydrocortisone (PerkinElmer NET396250UC) tracer. The inter- and intra-assay variations were < 10.0% and the lower limit of detection was 1.0 nmol/L.

5.2.3.2 Behavior at school

Teachers rated children's behavior on 119 items of the Teacher Report Form (TRF; Achenbach, 1991). Each item was rated on a 3-point scale (0 = *completely not applicable*, 2 = *clearly or often applicable*). Scores for *internalizing* and *externalizing* behavior were derived from the internalizing and externalizing subscales of the TRF. Cronbach's α was 0.88 and 0.93, respectively, for the internalizing and externalizing scale. To increase the normal distribution of the residuals of the regression analyses, scores for internalizing and externalizing behavior were log transformed.

Teachers rated children's prosocial behavior using a 10-item subscale of the Preschool Social Behavior Questionnaire (PSBQ; Tremblay, Vitaro, Gagnon, Piché, & Royer, 1992). Each item was rated on a 4-point scale (0 = *certainly not characteristic*, 3 = *very characteristic*). The subscale measures altruistic behavior with peers (Cronbach's α = 0.93). No transformation of scores was necessary to improve the normal distribution of the residuals of the regression analysis.

5.2.3.3 Parenting stress

Mothers filled in the Dutch version of the Parenting Stress Index (PSI; Abidin, 1983): the Nijmeegse Ouderlijke Stress Index (NOSI; de Brock, Vermulst, Gerris, & Abidin, 1992), a measure of parental experiences of stress due to the parenting situation. The NOSI has 123 items rated on a 6-point scale (1 = *completely disagree*, 6 = *completely agree*) measuring the domains of parental competence, role restriction, attachment, depression, health, social isolation, marital relationship, as well as child adaptability/plasticity, acceptability, demandingness, mood, distractibility/hyperactivity, and positive reinforce-

ment. Higher scores represented higher levels of maternal feelings of parenting stress with regard to the studied child. Cronbach's α for this measure was 0.96.

5.2.3.4 Confounders

Child gender and educational level of the mother were recorded as potential confounders. To measure mothers' educational level, they were asked to indicate their highest educational level ranging from 1 (*primary*) to 8 (*university*), followed by "*other*." Answers on this last option (n = 5) were recoded into the closest matching option. Gender was coded as *girl* (0) or *boy* (1).

5.2.4 Data Preparation

Of the 149 children, five were excluded from the analyses: one because of large deviations from the standard saliva sampling moments, one because of refusal to participate in the saliva sampling, and three because they used medication that might have influenced their cortisol concentrations. Cortisol concentrations of children who had a cold on the testing day (n = 7 of 144) did not differ from those of the other children (all p's > .050) and were therefore retained in the analyses.

5.2.4.1 Cortisol variables

In Figure 5.1, cortisol concentrations (nmol/L) and standard errors for each of the six sampling moments can be found. To analyze whether the stressor, the CREST, induced an increase from the baseline cortisol concentration (lowest of samples C1 and C2; e.g., de Veld, Riksen-Walraven, & de Weerth, 2012, 2014; de Weerth et al., 2013a) to the peak response concentration (highest of samples C3 and C4; de Weerth et al., 2013a), a paired samples t-test was conducted. There was a significant difference between baseline (M = 6.06, SD = 2.70) and peak response (M = 7.12, SD = 3.79) cortisol concentrations, $t(141) = -4.41$, $p < .001$, indicating that the paradigm induced a significant increase in children's cortisol concentrations.

Two common indices of cortisol stress responses were calculated: cortisol stress reactivity and total stress cortisol concentrations. There was a significant correlation between the baseline cortisol concentration and the peak response concentration, Spearman's *rho* = .69, $p < .001$. Therefore, children's cortisol stress reactivity scores were calculated as the standardized residuals of a regression of the peak response on the baseline score (de Veld et al., 2012; Schuetze, Lopez, Granger, & Eiden, 2008). Total stress cortisol concentrations across all 6 samples were calculated as the area under the curve: AUC = (C2 + C1) x 15/2 + (C3 + C2) x 10/2 + (C4 + C3) x 10/2 + (C5 + C4) x 15/2 + (C6 + C5) x 8/2.

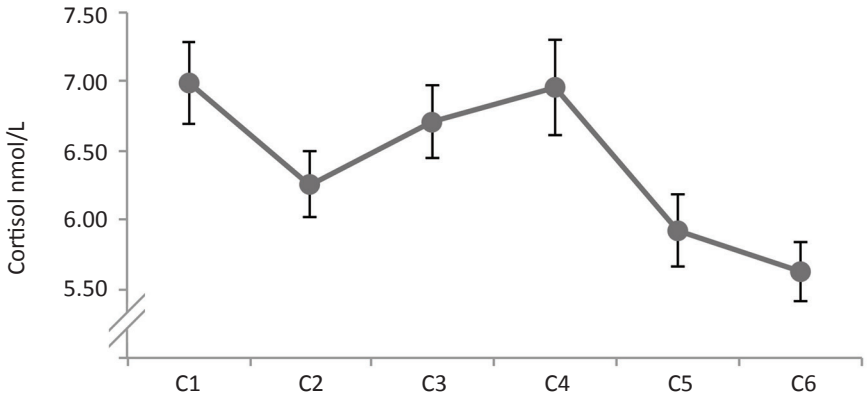

Figure 5.1 | Cortisol concentrations (nmol/L) per measurement moment. Error bars represent one standard error above and beneath the mean at each measurement moment.

5.2.4.2 Missing data

Of the 144 retained children, maternal parenting stress, was obtained for 139 children (3.5% missing) and behavior at school for 124 children (13.9% missing). Of the single cortisol samples 21 were missing (2.4%) and total stress cortisol concentrations and cortisol stress reactivity were calculated for 134 children (6.9% missing) and 142 children (1.4% missing), respectively.

5.3 Results

5.3.1 Preliminary Analyses

Children tested with the mobile laboratory parked near their home instead of their school (due to the parents' or schools' wishes; n = 7 of 144) did not differ significantly from the rest on outcome or predictor variables (all *p's* > .050). Hence, these children were included in the analyses. Descriptive statistics of the untransformed data are presented in Table 5.1.

Table 5.2 presents Spearman correlations of the predictor, outcome, and confounding variables. Higher maternal parenting stress was associated with less prosocial behavior (*rho* = -.20, p = .030). There was a significant correlation between child gender and prosocial behavior (*rho* = -.25, p = .005). Teachers rated girls as more prosocial than boys. Confounders that were significantly associated with an outcome variable were included in the pertaining regression analysis (Tabachnick & Fidell, 2007). Therefore, gender was included as a confounder in the regression predicting prosocial behavior.

Table 5.1 | *Descriptive Statistics of all Study Variables*

	N	*M*	*SD*	*Min*	*Max*
Confounders					
Child gender (% girls)	144	47.2%			
Educational level mother	140	6.78	1.39	3.00	8.00
Predictors					
Total stress cortisol (AUC)	134	375.80	169.48	73.80	1474.50
Cortisol stress reactivity[a]	142	0.00	1.00	-1.77	5.15
Parenting stress	139	229.22	54.24	144.00	426.00
Outcomes					
Externalizing behavior	124	5.73	7.79	0.00	35.00
Internalizing behavior	124	6.27	6.47	0.00	42.00
Prosocial behavior	124	13.79	6.30	1.00	30.00

Note. AUC = area under the curve (total stress cortisol concentration).
[a]Due to the operationalization of reactivity as standardized residuals the mean of this variable is 0.00 and the *SD* is 1.00.

Due to missing outcome or predictor variables 34 of the 144 children dropped out of the main regression analyses. These 34 children did not differ significantly from the other 110 on maternal educational level, gender of the child, or child age four temperament (CBQ short form; Putnam & Rothbart, 2006), all *p's* > .050. Mothers of children that dropped out were significantly younger (M_{age} = 37.48; *SD* = 4.34) than mothers of children remaining in the main analyses (M_{age} = 39.06; *SD* = 3.75), *t*(142) = -2.07, *p* = .040.

5.3.2 Main Analyses

A hierarchical regression analysis of prosocial behavior indicated that the first Step (including gender of the child) was significant, *F*(1, 108) = 4.05, *p* = .047. Gender of the child was significantly associated with prosocial behavior (Table 5.3). Teachers rated boys as less prosocial than girls. Step 2 of this regression (in which the two cortisol stress response indices were added) did not have significant additive value and the model itself was not significant (both *p's* > .050). Step 3 (in which maternal parenting stress was added) had significant additive value, F_{change} (1, 105) = 4.31, *p* = .040, and the model was significant, *F*(4, 105) = 2.98, *p* = .023. Within this model, maternal parenting stress was significantly associated with prosocial behavior (Table 5.3). Children of mothers with more parenting stress were seen as less prosocial by their teachers. Step 4, (adding the interaction terms between the cortisol stress response indices with maternal parenting stress) did not have significant additive value and the model was not significant (both *p's* > .050).

Table 5.2 | *Spearman Correlations Between all Study Variables*

	1.	2.	3.	4.	5.	6[a].	7[a].
Confounders							
1. Child gender							
2. Educational level mother	-.00						
Predictors							
3. Total stress cortisol (AUC)	-.04	-.05					
4. Cortisol stress reactivity	.10	-.12	.46***				
5. Parenting stress	.05	.14	-.02	-.07			
Outcomes							
6. Externalizing behavior[a]	.05	.00	-.05	-.03	.07		
7. Internalizing behavior[a]	.07	.06	-.18[+]	-.07	.14	.30**	
8. Prosocial behavior	-.25**	.07	.14	-.08	-.20*	-.40***	-.42***

Note. AUC = area under the curve (total cortisol concentration).

[a]log transformed.

[+] $p < .100$, * $p < .050$, ** $p < .010$, *** $p < .001$.

Regarding the other outcome variables (internalizing and externalizing behavior), none of the steps (adding the cortisol stress response indices in Step 1, maternal parenting stress in Step 2, and the interactions between the cortisol stress response indices with maternal parenting stress in Step 3) had significant additive value and none of the models were significant (see Table 5.3; all *p's* > .050). In other words, no significant associations were found of the cortisol stress responses, maternal parenting stress, or the interactions between the cortisol stress response indices and maternal parenting stress, with internalizing and externalizing behavior.

Table 5.3 | *Regression Models of Cortisol Stress Responses (Cortisol Stress Reactivity and Total Stress Cortisol Concentrations - AUC) and Maternal Parenting Stress on Behavioral Functioning at School*

	Model 1		Model 2		Model 3		Model 4	
	B	*ß*	*B*	*ß*	*B*	*ß*	*B*	*ß*
Externalizing behavior								
Step 1								
AUC			< 0.01	.02	< 0.01	.01	< 0.01	.06
Reactivity			-0.05	-.12	-0.05	-.11	-0.07	-.14
Step 2								
Parenting stress					< 0.01	.07	< 0.01	.04
Step 3								
AUC x Parenting stress							< -0.01	-.16
Reactivity x Parenting stress							< 0.01	.01
R^2_{change}			.01		.01		.02	
R^2_{model}			.01		.02		.04	

Note. AUC = cortisol area under the curve (total stress cortisol), reactivity = cortisol stress reactivity. No outliers were removed because Cook's distances indicated no potentially influential data points.

$^{+}$ $p < .100$, * $p < .050$.

Table 5.3 | *Regression Models of Cortisol Stress Responses (Cortisol Stress Reactivity and Total Stress Cortisol Concentrations - AUC) and Maternal Parenting Stress on Behavioral Functioning at School (continued)*

	Model 1		Model 2		Model 3		Model 4	
	B	*ß*	*B*	*ß*	*B*	*ß*	*B*	*ß*
Internalizing behavior								
Step 1								
AUC			< -0.01	-.03	< -0.01	-.04	< -0.01	-.01
Reactivity			-0.07	-.17	-0.06	-.15	-0.08	-.19
Step 2								
Parenting stress					< 0.01	.18^{+}	< 0.01	.17^{+}
Step 3								
AUC x Parenting stress							< -0.01	-.06
Reactivity x Parenting stress							< 0.01	.14
R^2_{change}			.03		.03^{+}		.01	
R^2_{model}			.03		.07^{+}		.08	
Prosocial behavior								
Step 1								
Child gender	-2.23	-.19*	-2.39	-.20*	-2.08	-.18^{+}	-1.97	-.17^{+}
Step 2								
AUC			0.01	.19^{+}	0.01	.20^{+}	0.01	.17
Reactivity			-0.96	-.16	-1.04	-.17	-0.97	-.16
Step 3								
Parenting stress					-0.02	-.19*	-0.02	-.18^{+}
Step 4								
AUC x Parenting stress							< 0.01	.08
Reactivity x Parenting stress							< 0.01	.01
R^2_{change}	.04*		.03		.04*		.01	
R^2_{model}	.04*		.06^{+}		.10*		.11^{+}	

Note. AUC = cortisol area under the curve (total stress cortisol), reactivity = cortisol stress reactivity. No outliers were removed because Cook's distances indicated no potentially influential data points.
$^{+}$ $p < .100$, * $p < .050$.

5.4 Discussion

This study investigated whether 6-year-old's cortisol stress responses were associated with their behavioral functioning at school. In addition, the moderating role of stress in the family environment, in the form of maternal parenting stress, was investigated. The stress paradigm increased children's cortisol concentrations. However, no support for associations between cortisol stress responses and behavioral functioning or a moderating role of parenting stress was found. Finally, children of mothers with more parenting stress were seen as less prosocial by their teacher.

The finding that the stress paradigm increased children's cortisol concentrations replicated earlier research. De Weerth et al. (2013a) showed that the CREST is a valid stressor triggering a modest cortisol stress response in children around the age of 5-6 (N = 42, M_{age} = 5 years and 8 months), while leaving room for individual differences. Our replication in a larger and slightly older sample (N = 144, M_{age} = 6 years and 1 month) strengthens the case that the CREST is an effective social evaluative stressor for this age group. Moreover, the CREST is considered an ecologically valid stressor for the school environment, given that it consists of social evaluation of the child's performance during challenging tasks, containing elements of unpredictability and uncontrollability. This, together with the paucity of effective stressors for this age group (de Weerth et al., 2013a; Gunnar, Talge, & Herrera, 2009), makes the CREST a promising tool for future stress response research.

The fact that we found no support for associations between cortisol stress responses and behavioral functioning is in line with research that found no associations between cortisol reactivity and mother-reported impulsivity, internalizing, and externalizing behavior of 4.5-year-olds (Spinrad et al., 2009), and with a meta-analysis concluding that cortisol reactivity was not associated with various forms of externalizing behavior in childhood and adolescence (Alink et al., 2008).

Together with these earlier findings, our results may be pointing at a potential dissociation between 6-year-old children's cortisol stress responses and behavioral functioning at school. Although our findings do not warrant this conclusion and more research is needed, speculatively, developmental processes could be behind such a dissociation. The cortisol circadian rhythm appears to continue to develop during childhood and adolescence (Shirtcliff et al., 2012; Simons, Beijers, Cillessen, & de Weerth, 2015). Similarly, children's cortisol reactions to stressors may still be developing during this period. Hence, children's cortisol stress responses may be less trait-like than those of adults (calibration of stress reactivity; see Boyce & Ellis, 2005).

Notwithstanding the above, the fact that we found no support for associations between children's cortisol stress responses and behavioral functioning in our study is not

in line with earlier findings that cortisol reactivity is associated with self-regulation, student-teacher relationships, school engagement, academic competence, externalizing behavior, aggression, and delinquency (Blair et al., 2005; Conradt et al., 2014; Obradović et al., 2010; Spinrad et al., 2009). Study characteristics may play a role in these apparently contradictory findings.

First, previous studies focused on various aspects of behavioral functioning that associated differently to cortisol responses. For example, Spinrad et al. (2009) reported associations of cortisol reactivity with effortful control, but not with impulsivity, internalizing, and externalizing behavior. This suggests that associations might be behavior-specific.

Second, studies differ in the observers of child behavior. Agreement between teachers and parents regarding child behavior is low to moderate (Winsler & Wallace, 2002). Behavioral functioning may be situation-specific, and teachers and parents may report more accurately on child behaviors in the context where they observe them most often (school vs. home). This may explain the differences between our findings with teacher reports, and those of Obradović et al. (2010), using a combined measure of parent, teacher, and self-reports.

Third, studies were conducted in different environments. Our sample was middle class whereas earlier studies examined children in less safe environments (e.g., Blair et al., 2005; Conradt et al., 2014; Obradović et al., 2010; Quellet-Morin et al., 2011). While there is ample evidence that (early life) environment affects the HPA-axis, the brain, and behavior (e.g., Kudielka et al., 2009; Loman & Gunnar, 2010; Lupien, McEwen, Gunnar, & Heim, 2009; McCarty et al., 2005; Pachter et al., 2006; Simons et al., 2015), the strength and type of associations between HPA-axis and behavioral functioning may be environmentally specific. This could explain why we found no support for associations whereas associations were found by Blair et al. (2005), Conradt et al. (2014) and Obradović et al. (2010).

The fact that our study was conducted with middle class families also suggests that children went to schools with relatively good educational support. This may have reduced the development of behavioral problems (note that in our study only 10.5% and 12.9% of the children scored above the clinical cut-off for internalizing or externalizing problems, respectively; Achenbach, 1991; Verhulst, van der Ende, & Koot, 1997). Protection from the development of behavioral problems may also reduce the formation of a link between behavioral functioning and cortisol stress responses.

The fact that we found no support for *moderation* by maternal reports of parenting stress is partly in line with Obradović et al. (2010). Although, Obradović et al. (2010) found an interaction effect of the cortisol stress responses and family adversity on prosocial behavior, they did not find this for three other aspects of behavioral functioning; externalizing behavior, school engagement, and academic competence. Although these findings

do not indicate the absence of the moderating role per se, they may suggest that family stress does not have a general effect on the association between cortisol stress responses and behavioral functioning. Regarding maternal parenting stress, it might be that it affects the child's experiences of stress in the environment less than expected. In line with this, Anthony et al. (2005) found that the association between parenting stress and child behavior was not mediated by parenting behavior. Stress in the family social environment may have more effect when it is more directly aimed at the child. And indeed, Quellet-Morin et al. (2011) found that lower cortisol responses were associated with more behavioral and social (not emotional) problems only in bullied and/or maltreated children.

Alternatively, differences in study characteristics may explain why we found no support for a moderating role of maternal reports of parenting stress. In addition to the characteristics described above, different operationalizations of environmental stress were used. We studied maternal parenting stress. Obradović et al. (2010) and Quellet-Morin et al. (2011) focused on environmental stressors such as financial stress, parenting overload, marital conflict, family expressiveness, maternal depression, maltreatment, and bullying. Some of these factors are also represented in parenting stress in our study (e.g., depression and marital relationship). However, the measures used by Obradović et al. (2010) and Quellet-Morin et al. (2011) also captured more severe forms of stress, potentially affecting the child more, uncovering a link between cortisol stress responses and behavioral functioning.

Interestingly, though not unexpected, children of mothers who experienced more parenting stress were seen as less prosocial by their teacher. This is in line with research indicating that a more adverse environment is associated with children's behavioral functioning (e.g., Conradt et al., 2014; McCarty et al., 2005; Pachter et al., 2006), that more parenting stress is associated with lower levels of child social competence (Anthony et al., 2005), and that parental positivity (positive feelings towards the child and non-coercive parenting) is positively associated with child prosocial behavior (Knafo & Plomin, 2006).

5.4.1 Strengths, Limitations, and Future Studies

Study assets were the use of an innovative, age-appropriate and ecologically valid stressor, which successfully increased cortisol concentrations, as well as the use of biological assessments and two sources of information (i.e., teacher and maternal report). A study limitation was the typical middle class sample as well as the fact that the correlational nature of the data precludes causal conclusions.

Future studies should include broader environmental contexts, also including less safe environments and non-Western cultures to increase generalizability. Moreover, the timing of stress in the environment may be important. Earlier research indicated that

stress early in life is associated with HPA-axis functioning of the child (e.g., Kudielka et al., 2009; Loman & Gunnar, 2010; Quellet-Morin et al., 2011; Simons et al., 2015). Potentially, stress in early life may have a more profound impact than stress later in life, or may have an additive effect upon current stress levels (Essex, Klein, Cho, & Kalin, 2002).

5.4.2 Conclusion

In this study, children of mothers who experienced more parenting stress displayed less prosocial behavior at school. However, support for associations between 6-year-olds' cortisol stress responses and teacher-reported internalizing, externalizing, and prosocial behavior was lacking, and there was no evidence for moderation by maternal parenting stress.

Chapter 6

General Discussion

In Section 6.1 to 6.3 of this general discussion insights regarding each of the three main research aims of this dissertation are discussed. Subsequently, early childhood as a developmental period for HPA-axis functioning will be addressed as well as possibilities to translate the obtained knowledge into clinical practice (Sections 6.4 and 6.5). Finally, strengths and limitations, suggestions for future research, and the main conclusions are addressed in Sections 6.6 to 6.8. In Table 6.1 a summary of the main findings for each research aim and empirical dissertation chapter is provided.

6.1 Dynamics and Development

The first aim of this dissertation was *to examine the dynamics and development of HPA-axis functioning in children up to/at the age of 6*. It was found that in 6-year-old children, higher total diurnal cortisol concentrations and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol concentrations (Chapter 2 - *Aim 1.1*). There was no evidence for associations of the cortisol circadian indices with cortisol stress reactivity to the social evaluative stress test (Chapter 2 - *Aim 1.1*). Furthermore, the cortisol circadian rhythm in children showed developmental changes between the ages of 1 and 6. Specifically, total diurnal cortisol concentrations declined between the ages of 1 and 6 and their day-to-day stability increased between the ages of 2.5 and 6 (Chapter 3 - *Aim 1.2*). There was no evidence for developmental change in diurnal cortisol decline or its day-to-day stability (Chapter 3 - *Aim 1.2*).

The finding that higher total diurnal cortisol and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol concentrations extends basic knowledge of HPA-axis functioning. It means that in typically developing 6-year-olds cortisol markers of diurnal HPA-axis functioning are associated with total stress cortisol. This may support the assumed associations between both patterns of cortisol production deriving from the same underlying mechanism, HPA-axis functioning. However, there was no evidence for associations between the circadian markers and cortisol stress reactivity in response to the social evaluative stress test. Future research may further extend this basic knowledge and indicate if in young school-age children the increase in cortisol in response to a stressor is independent of diurnal HPA-axis activity (as findings in this dissertation may suggest) or whether basal diurnal HPA-axis functioning inhibits or facilitates the ability to respond with an efficient increase in cortisol in an acute stressful situation.

Table 6.1 | *Main Findings per Research Aim and Empirical Chapter*

Chapter	Aim 1	Aim 2	Aim 3
	Dynamics and Development	*Behavior*	*Environment*
2	Higher total diurnal cortisol and smaller diurnal decline were uniquely associated with higher total stress cortisol. No evidence for a predictive role of circadian indices on cortisol stress reactivity. No evidence for a predictive role of the interaction of the circadian indices on the cortisol stress response indices.		
3	Total diurnal cortisol decreased from age 1 to 6 and its day-to-day stability increased between 2.5 and 6 years. No evidence for development of diurnal cortisol decline from age 1 to 6.		More maternal pregnancy-specific stress was associated with higher total diurnal cortisol. Lower levels of early postnatal maternal anxiety and higher levels of early postnatal maternal daily hassles were associated with steeper diurnal cortisol decline. No evidence for association of the interactions between maternal distress early in the child's life and age with the indices of the cortisol diurnal rhythm.
4		A negative association between cortisol stress reactivity and gazing was found. No evidence for an association between total stress cortisol and gazing behavior.	Less maternal fear of giving birth, higher prenatal maternal evening cortisol and more early postnatal maternal feelings of anxiety were associated with more total stress cortisol. No evidence for associations between maternal distress early in the child's life and gazing or cortisol stress reactivity.
5		No evidence for associations between cortisol stress responses and teacher reported behavioral functioning.	No evidence for a moderating role of current maternal parenting stress on the link between cortisol stress responses and teacher reported behavioral functioning.

The fact that total diurnal cortisol declined between the ages of 1 and 6 adds to our understanding of the development of the cortisol circadian rhythm. Although there is still a gap in knowledge regarding development of the cortisol circadian rhythm between the ages of 6 and 9, together with research by Shirtcliff et al. (2012), Saridjan et al. (2010), Watamura et al. (2004), and research during the first year of life (e.g., Custodio et al., 2007; de Weerth & van Geert, 2002; de Weerth, Zijl, & Buitelaar, 2003; Ivars et al., 2015; Spangler, 1991), the findings of this dissertation suggest that the cortisol circadian rhythm continues to develop during childhood and adolescence. Future longitudinal research may want to investigate if developmental changes takes place equally during each age period or whether specific periods are characterized by specific developmental changes.

The decline in total diurnal cortisol concentrations between the ages of 1 and 6 indicates that development in this period should be taken into account when comparing total diurnal cortisol concentrations between cross-sectional studies with different age groups, and when looking at total diurnal cortisol concentrations for diagnostic purposes. Moreover, it underscores the need to consider, and if required take age into account, in research designs (e.g., Nicolson, 2007) including this biomarker in children. This can be done by narrowing the age range of participants in a sample or by including age as a control variable in the analyses.

The finding that the day-to-day stability of total diurnal cortisol increased between 2.5 and 6 years (Chapter 3) suggests that reliable measures of this marker can be obtained across fewer measurement days in older than in younger children. In line with this, in 3- to 7-month-old's day-to-day stability is low (Spangler, 1991) and de Weerth and van Geert (2002) found that large inter-individual variability in 5- to 8-month-old children made it difficult to increase the reliability of basal diurnal measures by adding more measurement days. In other research, intra-individual variability of cortisol samples taken at 11 AM decreased during the second half year of life (Tollenaar, Jansen, Riksen-Walraven, Beijers, & de Weerth, 2010). Although in this dissertation no evidence for developmental change in day-to-day stability of total diurnal cortisol between the ages of 1 and 2.5 and in diurnal cortisol decline between the ages of 1 and 6 was found, the studies by de Weerth and van Geert (2002), Spangler (1991), and Tollenaar et al. (2010) suggest that the principle of obtaining reliable aggregated total diurnal cortisol measures with fewer measurement days in older than in younger children may also apply to a somewhat younger age group than studied in this dissertation, and to cortisol samples taken at specific times during the day.

Intra-individual variability/stability and/or the number of aggregated sampling days needed to reliably measure cortisol markers have been studied for various markers and ages in several ways resulting in various observations and/or recommendations (e.g., de Weerth & van Geert, 2002; Hruschka, Kohrt, & Worthman, 2005; Kraemer et al., 2006;

Oskis, Loveday, Hucklebridge, Thorn, & Clow, 2009; Pruessner et al., 1997; Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012; Sergerstrom, Boggero, Smith, & Sephton, 2014; Tollenaar et al., 2010). Age differences in day-to-day stability of cortisol markers during early childhood were not always taken into account in recommendations regarding the number of sampling days needed to obtain reliable markers. Future research taking age differences in day-to-day stability during early childhood into account may help to increase the ability to determine the ideal number of sampling days to obtain optimally reliable diurnal cortisol estimates in children.

Apart from age differences in day-to-day stability, logistics and subject burden should also be taken into account in considerations regarding the number of sampling days to collect (e.g., Rotenberg et al., 2012). Although collecting cortisol during more sampling days may be regularly recommended, especially when investigating diurnal decline, this is not always possible in vulnerable participants such as children. In Chapter 3, the correlations between the markers of the cortisol circadian rhythm across the two sampling days were (marginally) significant and weak to moderate at ages 1 and 2.5 (r = .21 to r = .38) and significant and moderate to strong at the age of 6 (r = .30 and r = .58). This suggests that especially the findings of Chapter 3 regarding the development of the cortisol circadian rhythm and its predictors might have been affected if cortisol diurnal samples had been aggregated over more sampling days. Future studies should further examine the stability of the patterns found.

6.2 Behavior

The second aim of this dissertation was *to examine the associations between HPA-axis functioning and behavior of children at the age of 6.* The studies indicated that cortisol stress reactivity and gazing behavior during an acute social stressful situation were associated (Chapter 4 - *Aim 2.1*). There was no evidence for an association between total stress cortisol and gazing during the stressor (Chapter 4 - *Aim 2.1*) or associations between cortisol stress responses and behavioral functioning in the classroom (Chapter 5 - *Aim 2.2*).

These findings may suggest that cortisol markers of HPA-axis functioning and behavior are associated within a situation, but not necessarily across situations. This leads to the hypothesis that whereas cortisol and behavioral reactivity in stressful situations are associated (as found in Chapter 4), the basal diurnal cortisol rhythm may be associated with more basic, general behavioral functioning during the day. Additional exploratory analyses were performed to test this idea.

Specifically, to test the association between the basal diurnal cortisol rhythm and behavioral functioning during the day, similar analyses were conducted as those in Chapter 5, this time using the cortisol circadian indices instead of the cortisol stress response indices as markers of HPA-axis functioning. These analyses yielded a significant regression model predicting prosocial behavior from gender, total diurnal cortisol, and diurnal cortisol decline, $F(3, 97) = 4.98$, $p = .003$, $R^2 = .13$. Teachers rated the level of prosocial behavior lower amongst boys than girls ($\beta = -.27$, $p = .005$). Higher child total diurnal cortisol ($\beta = .29$, $p = .023$) and a smaller diurnal decline ($\beta = -.36$, $p = .006$) were both uniquely associated with more teacher-reported prosocial behavior. No evidence for associations with teacher-reported internalizing or externalizing behavior was found. Moreover, an additional exploratory analysis indicated no evidence for associations between the indices of the basal cortisol circadian rhythm and gazing behavior during the social evaluative stressful situation. The associations between indices of the basal diurnal cortisol rhythm and everyday prosocial behavior partially support a link between the diurnal cortisol rhythm and behavioral functioning during the day. It should be noted that in this dissertation the cortisol circadian rhythm was measured on days that the child did not attend childcare or school whereas behavioral functioning was reported by the teacher. Since children's cortisol diurnal patterns differ between weekend- and school-days (Gutteling, de Weerth, & Buitelaar, 2005), hypothetically, links between circadian markers and behavioral functioning on similar days may be stronger.

A meta-analysis of the associations of cortisol stress reactivity and basal cortisol concentrations with externalizing behavior (Alink et al., 2008) also supports part of the hypothesis that cortisol and behavioral reactivity in stressful situations are associated, whereas the basal diurnal cortisol rhythm may be linked to more basic, general behavioral functioning during the day. Alink et al. (2008) found no support for links between cortisol stress reactivity and externalizing behavior, but links were found between basal cortisol concentrations and externalizing behavior. The meta-analysis of Alink et al. (2008) also included clinical samples, and yielded a link for all samples together and for the clinical samples separately but not for the non-clinical samples separately. Our study included a non-clinical sample only. This may explain the difference between our findings and the meta-analysis by Alink et al. (2008). Although findings of Alink et al. (2008) are in line with the hypothesis that whereas cortisol and behavioral reactivity in stressful situations are associated, the basal diurnal cortisol rhythm may be linked to more basic, general behavioral functioning during the day, Klimes-Dougan, Hastings, Granger, Usher, and Zahn-Waxler (2001) found that in adolescence both the circadian rhythm and the cortisol stress response were associated with internalizing behavior.

Together, the above partially supports the hypothesis that cortisol markers of HPA-axis functioning and behavior are associated within a situation, but not necessarily across situations. However, more research is needed to examine this hypothesis further.

In this dissertation children's behavior was measured using two different "observers". Internalizing, externalizing, and prosocial behavior was operationalized using questionnaires about general behaviors filled out by the teacher. Gazing behavior during the social stressor was observed using detailed behavioral observations by researchers. In future research, using various behavioral observers in various (social) contexts, may broaden the picture of associations between children's HPA-axis functioning and behavior.

6.3 Environment

The third aim of this dissertation was *to examine the role of environmental stress (early) in children's lives on behavior during stressful situations and (the development of) HPA-axis functioning, as well as on the associations between HPA-axis and behavioral functioning up to/at the age of 6*. Maternal distress early in children's lives (prenatal and early postnatal) was associated with child cortisol markers of later HPA-axis functioning (Chapters 3 and 4 - *Aim 3.1 and 3.2*). There was no evidence for a moderating role of current maternal parenting stress on the link between 6-year-olds' cortisol stress responses and their behavioral functioning at school (Chapter 5 - *Aim 3.3*).

The associations between maternal prenatal and early postnatal distress and child cortisol indices of HPA-axis functioning support the idea of early programming and of stress early in life affecting later HPA-axis functioning (e.g., Chaby, 2016; Kudielka, Hellhammer, & Wüst, 2009; Loman & Gunnar, 2010; Lucas, 1991). The fact that associations between maternal distress and indices of child HPA-axis functioning were found in a sample of typically developing middle class children of relatively highly educated families living in safe environments stresses the importance of studying HPA-axis functioning in such a population. Associations may be even stronger in populations facing more (environmental) stress early in life (e.g., Shonkoff et al., 2012).

Interestingly, specific aspects of maternal distress were associated differently with different markers of child HPA-axis functioning and associations were not always strong (Chapter 3 and 4). The associations that were found are summarized in column 4 of Table 6.1. Such diverse findings have been found in numerous studies (e.g., Gutteling, de Weerth, & Buitelaar, 2004; 2005; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011). This points at the need to go beyond the specific associations and look at underlying mechanisms that link maternal distress to child development and specifically

HPA-axis functioning. A better understanding of the underlying mechanisms will help to explain the diverse findings.

During the prenatal phase various mechanisms may play a role in transferring maternal distress to the child (see, for reviews, Beijers, Buitelaar, & de Weerth, 2014; Rakers et al., 2017). As described in Chapters 3 and 4, mechanisms may for example be maternal cortisol concentrations or maternal health-related behavior that may impact the maternal immune system and placenta. Maternal distress can affect mothers' cortisol concentrations and their health-related behavior such as eating and sleeping (Beijers et al., 2014). This in turn may affect the physiology of the mother resulting in a less healthy, more stressful environment for the unborn child, affecting its developing HPA-axis (Beijers et al., 2014). This may occur, for example, via changes in the 11 ß-hydroxysteroid dehydrogenase-type 2 (11 ß-HSD2) enzyme (O'Donnell et al., 2012). Maternal distress may affect placental functioning, including a reduction of the 11 ß-HSD2 enzyme that protects the fetus by reducing the amount of maternal cortisol that crosses the placenta (e.g., Beijers et al., 2014; Rakers et al., 2017). Recently, additional biological mechanisms involving catecholamines, cytokines, and serotonine/tryptophan have also been described (see Rakers et al., 2017).

Another mechanism of how maternal distress may affect the child involves intestinal microbiota (e.g., Beijers et al., 2014; Rakers et al., 2017). Maternal distress may affect mothers' microbial composition which in turn may affect the colonization of the intestine of the newborn, affecting its development and that of the HPA-axis. A suggestion for this idea can be found in a study by Zijlmans, Korpela, Riksen-Walraven, de Vos, and de Weerth (2015b). They showed that maternal prenatal distress was associated with infants' microbial composition. Infants of mothers with more distress during pregnancy had microbial signatures that are associated with increased levels of inflammation. This may in turn affect the child's developing stress system. Studying the role of the intestinal microbiota seems to be a promising next step in understanding associations between maternal distress and child HPA-axis functioning or children's development more generally (e.g., Beijers et al., 2014; Rakers et al., 2017).

As described in Chapters 3, 4, and 5, postnatal maternal distress may, for example, be transferred to the child via maternal behavior. For instance, parenting stress was associated with parental self-reports of more discipline, less nurturing, and being laxer and less sensitive to the child (e.g., Anthony et al., 2005; Guajardo, Snyder, & Petersen, 2009; Pereira et al., 2012). Also mothers with an anxiety disorder were less sensitive towards their infant than healthy controls (Feldman et al., 2009). This in turn may affect the developing child's stress system since adverse caregiving can be a stressor for the child (see Loman & Gunnar, 2010). In line with this, parental responsivity at child age 4

(Hackman et al., 2013) and positive parenting in grade 8 (Shirtcliff, Skinner, Obasi, & Haggerty, 2017) were both predictive of children's cortisol stress response in adolescence. In addition, mechanisms that played a role during the prenatal phase, such as maternal health-related behaviors, may also continue to play a role postnatally, affecting the child's environment and/or development postnatally and in turn its HPA-axis.

The various types (or combinations) of maternal distress may affect the child through different underlying mechanisms, or routes within a mechanism (see also Beijers et al., 2014). This may explain the diverse associations between maternal distress and child HPA-axis functioning. For example, one form of maternal distress may affect the gut microbial compositions of the mother and in turn the child and his or her HPA-axis, whereas another form may be linked to maternal health-related behavior affecting the child's environment and in turn his or her HPA-axis.

Support for the idea that different forms of distress may affect children via different mechanisms may come from research showing links between specific aspects of maternal distress and maternal or child outcomes. For example, Lobel et al. (2008) indicated that from various forms of maternal distress (anxiety, perceived stress, life events, pregnancy-specific stress, or a combination of the four) especially pregnancy-specific stress predicted infant birth outcomes (i.e., weeks of gestation). Pregnancy-specific stress in turn was associated with maternal health-related behaviors such as smoking, unhealthy eating, and low physical activity (Lobel et al., 2008), suggesting that health-related behavior may be an underlying mechanism of this link that potentially also affects the child's development and HPA-axis. As described in Chapter 3 this may explain the association found between maternal pregnancy-specific stress and child total diurnal cortisol. Zijlmans et al. (2015b) indicated that associations between different aspects of maternal distress and infant microbial compositions varied in strength. From various maternal self-reported distress measures (anxiety, pregnancy experiences, daily hassles, fear of giving birth, and fear of bearing a handicapped child) the strongest association with infant microbiota was found for fear of bearing a handicapped child. As described above, a higher total score of maternal distress in turn was associated with infant microbial signatures associated with increased levels of inflammation. This suggests that the microbial composition from mother and child may be an underlying route of this link, possibly also affecting the child's HPA-axis. However, no evidence for a link between fear of bearing a handicapped child and child HPA-axis functioning was found in this dissertation.

Speculatively, the existence of various routes within a mechanism also may explain the diversity of findings. In other words, within the mechanism of transferring maternal distress to maternal behavior and in turn to the child's environment and the child, diverse links between the types of maternal distress and behavior are possible, affecting

the child differently. For example, some aspects of maternal distress may result in more structured, balanced, or protective maternal behavior; others may result in more chaotic behavior and/or an inability to protect the child from environmental stressors. The former results in a less stressful environment for the child, the latter in a more stressful environment, affecting the child's HPA-axis differently.

Studying the mechanisms underlying the links between various types of maternal distress (or combinations thereof) and child development is an important next step in research (e.g., Beijers et al., 2014; Glover, Ahmed-Salim, & Capron, 2016). This will help to clarify the variability in associations between maternal distress and child HPA-axis functioning. It may also provide entries for developing interventions targeting specific links between maternal distress and child development. Moreover, as described in Chapter 3 and 4, the role of genetic factors and shared environment should also be taken into account in future research examining links between maternal distress and child HPA-axis functioning.

6.4 Early Childhood

Total diurnal cortisol concentrations declined between the ages of 1 and 6 and their day-to-day stability increased between the ages of 2.5 and 6 (Chapter 3). Moreover, there were associations between maternal prenatal and early postnatal distress and cortisol indices of HPA-axis functioning (Chapters 3 and 4; see Table 6.1, column 4 for the associations found). This indicates that the period between the ages of 1 and 6 is an important developmental period for HPA-axis functioning.

Although no evidence for changes in cortisol diurnal decline day-to-day stability was found, the fact that day-to-day stability of total diurnal cortisol increased between 2.5 and 6 years (Chapter 3) may indicate that the cortisol circadian rhythm consolidates during this period. In an overview paper, Boyce and Ellis (2005) already suggested that stress reactivity may become calibrated and more canalized, more resistant to change, and decreasing in plasticity during childhood. Speculatively, HPA-axis functioning in general becomes more consolidated and less influenced by the environment during childhood. In line with this, Belsky and Pluess (2013) and Loman and Gunnar (2010) suggested that environmental effects (on the developing stress neurobiology) may be the largest early in life.

Various studies looked at the timing of environmental effects on child HPA-axis functioning. For example, in line with the idea of the importance of early life, Laurent (2017) found that of maternal depression measured at 3, 6, 12, and 18 months postnatally, particularly the severity of a clinical depression at 3 months was associated with the infants' cortisol stress response. McLaughlin et al. (2015) indicated that increasing the quality of early life caregiving by adoption from deprived institutionalized care positively affected

the HPA-axis response to stressors only when adoption took place before the age of 24 months. However, not all studies support the importance of early life as some find that adversity in childhood and adolescence may be especially influential for later HPA-axis functioning (Pesonen et al., 2010; Bosch et al., 2012).

Additionally, Belsky and Pluess (2013) hypothesized that individuals may differ in when they are most susceptible to environmental factors. Hypothetically this may also apply to the developing HPA-axis and different environmental factors may have a different impact during different developmental stages. These ideas should be further investigated because they may have implications for understanding at what ages children are most susceptible to which environmental input. This may also have implications for intervention or prevention possibilities at certain ages.

As described earlier, developmental changes in the cortisol circadian rhythm were found and it is possible that also the cortisol stress response (i.e., HPA-axis functioning in general) shows developmental changes in childhood. Indeed, Laurent (2017) found a longitudinal increase in reactivity dynamics and a reduction in total cortisol output to stressors between the ages of 6 and 18 months. Moreover, Leppert, Kushner, Smith, Lemay, and Dougherty (2016) found longitudinal changes in the cortisol stress responses from early to middle childhood.

Leppert et al. (2016) and Laurent (2017) used different social stressors for each measurement moment. On the one hand, using different stressors makes it impossible to rule out the nature of the stressor as an alternative explanation. Using the same stress protocol at different ages would allow the most direct and controlled comparison of stress responses across age. On the other hand, repeated stress exposure may result in learning and habituation (e.g., de Weerth, Buitelaar, & Beijers, 2013b; Wüst, Federenko, van Rossum, Koper, & Hellhammer, 2005), possibly also depending on the age of the child and the time period between the two stress exposures. Moreover, different stimuli are experienced as stressful at different ages, making it impossible to use the same stressor from birth into adolescence (Gunnar, Talge, & Herrera, 2009). Nonetheless studies using both the same and different but comparable age-appropriate stressors within the same sample over time are important to understand the longitudinal development of cortisol stress responses during early childhood.

6.5 Implications for Clinical Practice

An important question is whether and how the knowledge obtained in this dissertation can be used in clinical practice. As stated in the introduction, knowledge of developmen-

tal changes in the cortisol circadian rhythm may be useful for diagnostic purposes. Understanding the associations between cortisol stress responses and gazing in a stressful situation may be a first step to detect opportunities for training children in behavioral strategies to manage cortisol stress responses. Also, knowledge of the links between children's environment and the cortisol circadian rhythm and stress response may provide entries for interventions. In line with this last idea, a systematic review by Slopen, McLaughlin, and Shonkoff (2014) and research by McLaughlin et al. (2015) indicated that psychosocial interventions can modify cortisol indices of HPA-axis functioning of children.

However, to bridge fundamental knowledge and clinical practice it is necessary to know what can be seen as normative versus extreme and to keep in mind what can be seen as adaptive for the child. Earlier research investigated reference values of saliva cortisol concentrations at various ages and for various markers of the circadian rhythm (e.g., Aardal & Holm, 1995; Hansen, Garde, Christensen, Eller, & Netterstrøm, 2003; Kobayashi & Miyazaki, 2015; Mert et al., 2013; Rolfsjord et al., 2017; Tollenaar et al., 2010; Törnhage, 2002; Wüst et al., 2000). However, these studies did not provide a picture of the total diurnal cortisol pattern in young children. Recently, Ivars et al. (2015) described references values of morning, noon, and evening cortisol in infants between the age of 1 and 12 months and Miller et al. (2017) conducted a large scale study on normative cortisol circadian concentrations at 7 time points during the day across the lifespan.

Although this knowledge of reference scores for diurnal cortisol concentrations at specific time points during the day is very useful, no age differentiations were made within the periods of 1 to 5 years, and 5 to 10 years. The findings of this dissertation indicate that the cortisol circadian rhythm, specifically total diurnal cortisol concentrations, shows developmental change between the ages of 1 and 6 (Chapter 3). This suggests the need to take age within these groups into account when developing reference values for total diurnal cortisol concentrations and potentially also for diurnal cortisol concentrations during specific times during the day. Reference scores for cortisol stress responses for various stressors would also be helpful to bridge fundamental knowledge and clinical practice. These should also be age-specific as development in cortisol stress responses is suggested (Laurent, 2017; Leppert et al., 2016) and not all stressors work equally well at each age (Gunnar et al., 2009). Moreover, it should be noted that the nature of the laboratory analyses should be take into account when comparing cortisol concentrations (with reference values) since these affect the obtained scores.

To bridge fundamental knowledge and clinical practice it is further helpful to keep in mind what can be seen as adaptive for the child and what are relevant thresholds for cut-off scores. Taking the context and knowledge of outcomes of certain patterns of HPA-axis functioning into account is important in this regard. According to the Adaptive Calibration

Model, the stress response system of an individual develops to be in line with her or his environment (Del Giudice, Ellis, & Shirtcliff, 2011). A stressful environment early in life can thereby regulate the stress system to develop in a way that results in adaptive functioning in such an environment. However, these physiological adaptations do not necessarily imply psychological well-being or other valued outcomes for the child (Del Giudice et al., 2011). Applying this to HPA-axis functioning means that although altered HPA-axis functioning may be seen as negative due to associations with health problems, it may at the same time be a functional adaptation that may be valuable for a child in another domain. For example, "altered" HPA-axis functioning resulting from a stressful environment may facilitate the child's survival in this environment by making the child more or less responsive to the situation, even though it has at the same time negative consequences for health. This means that taking the environment and outcomes in different domains into account is important when thinking about valence inferences of HPA-axis functioning.

Future research combining these aspects will help to broaden the understanding of HPA-axis functioning in children and will help to bridge fundamental knowledge and clinical practice regarding HPA-axis functioning.

6.6 Strengths and Limitations

As described in Chapters 2 to 5, strengths of the studies of this dissertation were for example the relatively large longitudinal sample, the combination of physiological, behavioral, and psychological measures, and the use of an effective stress test for 6-year-olds (de Weerth, Zijlmans, Mack, & Beijers, 2013a). Several limitations were also described in Chapters 2 to 5. Two specific (additional) limitations will be mentioned here.

First, cortisol recovery as a separate construct was beyond the scope of this dissertation. Although cortisol concentrations during the stress recovery phase were part of the operationalization of total stress cortisol, this was not studied separately. Cortisol recovery is mostly biologically determined, based on the half-life of cortisol, and hence is often seen as dependent on reactivity and not studied separately (see Dickerson & Kemeny, 2004; Linden, Earle, Gerin, & Christenfield, 1997). However, Dickerson and Kemney (2004) found that although cortisol peak responses predicted the effect sizes of cortisol recovery, they did not explain the total variance in recovery time. Moreover, a meta-analysis indicated that depressive participants differed from controls on recovery cortisol concentrations whereas no differences between the two groups were found in stress cortisol concentrations (Burke, Davis, Otte, & Mohr, 2005). This suggest that in some cases cortisol recovery may differentiate individuals more than cortisol reactivi-

ty. Moreover, altered cortisol recovery can result in prolonged exposure to heightened cortisol concentrations (McEwen, 2000) and cortisol recovery has been associated with health risks (e.g., Roy, Kirschbaum, & Steptie, 2001). Therefore, future studies of cortisol recovery to stressors during childhood may provide valuable additions to our knowledge. Studies investigating recovery should thereby preferably include cortisol measures during an extensive period after the stressor since in adults effect sizes of increased cortisol after motivational performance tasks including social evaluation and uncontrollability remain significant 41 to 60 min post stressor (Dickerson & Kemney, 2004).

As shortly described in Chapter 4 a second limitation is that in this dissertation cortisol stress responses were studied using a specific stressor. The stressor was innovative, age-appropriate, ecologically valid, and successful in increasing cortisol concentrations (de Weerth et al., 2013a). But in adults there is intra-individual variation in stress responses to different stressors (e.g., Kirschbaum, Wüst, Faig, & Hellhammer, 1992), and not all stressors work equally well in triggering an increase in cortisol in children (Gunnar et al., 2009). Thus, cortisol stress responses to our stress test, a social evaluative stress test including motivational performance and uncontrollability, most probably do not fully generalize to other stressors. Studying the links with stress responses to various ecologically-valid stressors will broaden our understanding of HPA-axis functioning.

This also points at a challenge for future research. The laboratory stress test of this dissertation is one of the few that is effective in increasing cortisol in this age group (de Weerth et al., 2013a; Gunnar et al., 2009). Developing additional effective laboratory stressors in this age group will facilitate studying links with stress responses to various ecologically-valid stressors. An example of such a test is a motivated performance matching task including forced failure and negative social evaluation, recently developed by Roos et al. (2017) resulting in significant cortisol increases in 4- to 6.7-year-old's.

6.7 Future Research

Future research can further broaden our understanding of HPA-axis functioning, its dynamics, development, predictors, and correlates. In Chapters 2 to 5 various ideas are mentioned. This section provides five additional suggestions.

First, as stated in Chapter 2, the use of four cortisol markers of HPA-axis functioning together representing both the cortisol stress response and the cortisol circadian rhythm provides insight in HPA-axis functioning. However, it is well-known that cortisol is a peripheral measure that provides a partial window on HPA-axis functioning (e.g., Egliston, McMahon, & Austin, 2007; Nicolson, 2007). Therefore, combining measures of

saliva cortisol with other indicators of HPA-axis functioning will further broaden our understanding of HPA-axis functioning. Developing valid, non-invasive measurement techniques to (indirectly) determine concentrations of the corticotrophin releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH) in children would be a valuable step for further research. However, the possibility to develop such measures for higher order processes in children is questioned (Doom & Gunnar, 2013).

Second, until now mothers are often studied as the primary "environment" in children's early life. Although in the first 6 months of life mothers are often the primary caregiver, fathers also play an important role. Since adverse caregiving may be a stressor for the child (Loman & Gunnar, 2010), postnatal paternal caregiving behavior can be expected to affect the child in a similar fashion as maternal caregiving behavior. Moreover, one study demonstrated that exposing male rats to stress during puberty or adolescence affected their germ cells and sperm MicroRNA and their offspring's HPA-axis functioning (Rodgers, Morgan, Bronson, Revello, & Bale, 2013). In humans, paternal distress before conception may also program the offspring's development and HPA-axis (see Braun, Messerlian, & Hauser, 2017). In addition, research with humans has shown that women's experience of partner support during pregnancy was associated with their infants' HPA-axis functioning in stressful situations at 6 months of age (Thomas et al., 2017). Maternal prenatal depressive feelings and the postnatal quality of mother-infant interactions were part of the pathway between maternal feelings of partner support and infant HPA-axis functioning (Thomas et al., 2017). Together, these findings indicate a strong need to include fathers and consider how they, directly and indirectly, affect children's environment and HPA-axis functioning.

Third, knowledge of the effects of environmental stress will also be broadened by using additional measures of maternal distress and/or environmental stress such as partner reports, experience sampling/diary data, observational data, auditory recordings of (parental) arguments or fights (see Slatcher & Robles, 2012), the amount and quality of care, or measuring chronic stress with hair cortisol (Smy et al., 2016; Stalder & Kirschbaum, 2012). Other aspects of maternal distress (e.g., depression (see Laurent, 2017) or stress due to major life events) will also broaden the research scope on environmental stress.

Fourth, child characteristics, such as self-regulation abilities, ego-resiliency, and temperament are of interest for future research on HPA-axis functioning. Although in earlier research not much support was found for associations between personality characteristics and cortisol stress reactivity (see Kudielka et al., 2009), exploring the role of other child characteristics is valuable. Child characteristics may be directly associated with HPA-axis functioning. For example, effortful control was associated with cortisol concentrations in 12- to 36-month-olds (Watamura, Donzella, Kertes, & Gunnar, 2004)

and cortisol stress responses in 7-year-olds (Mayer, Abelson, & Lopez-Duran, 2014). Children scoring higher on ego-resiliency, reflecting higher abilities to cope with stressful situations, had lower cortisol responses to such a situation (Smeekens, Riksen-Walraven, & van Bakel, 2007). Moreover, child characteristics such as temperament may moderate associations between environmental factors and HPA-axis functioning (e.g., Kamin, Liu, Bhatt, Kelly, & Kertes, 2016), or determine links between HPA-axis functioning and behavior. In summary, including the role of child characteristics will broaden our understanding of HPA-axis functioning and may provide opportunities for intervention and prevention programs in vulnerable populations.

Finally, cortisol stress responses are often measured using exposure to a single novel stressor. The physiological reaction to such a stressor provides an indication of the stress response. However, the fact that it is limited to a single reaction to a stressor may reduce the generalizability to repeated stress exposure, for example when being exposed repeatedly to the same stressful situation in everyday life or to a chronically stressful environment. A lack of adaptation can be seen as an indication of altered HPA-axis functioning (McEwen, 2000). Moreover, cortisol stress response habituation has been associated with immune system functioning (e.g., Thoma et al., 2017) and associations with self-reported physical and mental symptoms have been reported (Kirschbaum et al., 1995). In addition, individual differences in infant habituation were associated with maternal prenatal stress (de Weerth et al., 2013b), and habituation of cortisol reactivity differentiated newborns who had suffered from stress early in life from those that had not, whereas no support for a differentiating role of the reaction to the first stressful situation was found (Gunnar, Hertsgaard, Larson, & Rigatuso, 1991). Together, these findings suggest that studying altered patterns of cortisol responses to repeated stress exposure may reveal additional insights in HPA-axis functioning.

6.8 Conclusions

Together, the studies in this dissertation indicate that total diurnal cortisol concentrations show developmental changes between the ages of 1 and 6 years, and that at the age of 6 indices of the cortisol circadian rhythm are associated with total stress cortisol concentrations. Moreover, maternal distress early in children's lives is associated with cortisol indices of their later HPA-axis functioning. Finally, gazing in response to a social stressful situation was associated with cortisol stress reactivity. Together, the results of this dissertation indicate that the period between the ages of 1 and 6 is an important developmental period for HPA-axis functioning.

Summary

A major player of the human stress system is the hypothalamic-pituitary-adrenal (HPA) axis that produces cortisol as its primary hormonal end product (e.g., Lupien, McEwen, Gunnar, & Heim, 2009; Nicolson, 2007). Alterations in cortisol markers of HPA-axis functioning have been associated with mental and physical health (e.g., Bremmer et al., 2007; Burke, Davis, Otte, & Mohr, 2006; Buske-Kirschbaum et al., 2003; Hankin, Badanes, Abela, & Watamura, 2010; Jessop & Turner-Cobb, 2008; Luby et al., 2003; McEwen, 2008; Phillips, Ginty, & Hughes, 2013; Sephton & Spiegel, 2003). These links make it important to understand HPA-axis functioning, its dynamics, development, predictors, and correlates. This is especially relevant in childhood since HPA-axis functioning seems to be developing (e.g., Boyce & Ellis, 2005; Saridjan et al., 2010; Shirtcliff et al., 2012; Watamura, Donzella, Kertes, & Gunnar, 2004), can be modified by interventions (e.g., McLaughlin et al., 2015; Slopen, McLaughlin, & Shonkoff, 2014), and is susceptible to environmental factors (e.g., Beijers, Riksen-Walraven, & de Weerth, 2013a; Chaby, 2016; Fernald, Burke, & Gunnar, 2008; Hunter, Minnis, & Wilson, 2011; Loman & Gunnar, 2010; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2012; van der Vegt, van der Ende, Kirschbaum, Verhulst, & Tiemeier, 2009). Understanding HPA-axis functioning during childhood may hence offer entries for designing future prevention and intervention programs targeting children early in life.

There are still a number of gaps in our knowledge of HPA-axis functioning in childhood including unanswered questions about the dynamics and development of HPA-axis functioning, the associations between HPA-axis functioning and behavior, and the effect of environmental stress (early) in children's lives on the HPA-axis, on behavior, and on the associations between HPA-axis and behavioral functioning. To address these issues, this dissertation focused on the following three overarching research aims specified in seven specific research aims:

1. To examine the **dynamics and development** of HPA-axis functioning in children up to/at the age of 6 (Specific aims 1.1 & 1.2).
2. To examine the associations between HPA-axis functioning and **behavior** of children at the age of 6 (Specific aims 2.1 & 2.2).
3. To examine the role of **environment**al stress (early) in children's lives on behavior during a stressful situation and (the development of) HPA-axis functioning, as well as on the associations between HPA-axis and behavioral functioning up to/at the age of 6 (Specific aims 3.1, 3.2, & 3.3).

All research aims were pursued in the BIBO project (Radboud University). BIBO is an ongoing prospective longitudinal project following a cohort of typically developing chil-

dren from middle class families from late pregnancy on. In this dissertation data from the prenatal period and the first 6 postnatal years were used, combining behavioral (observations and questionnaires), psychological (questionnaire), and biological (hormonal) measures. In the remainder of this chapter a summary of the four empirical chapters of the dissertation is provided followed by a general conclusion.

Chapter 2

The goal of this chapter was *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their cortisol circadian rhythm (Aim 1.1)*. To examine this, data of 149 6-year-old children who participated in a social evaluative stress test (Children's Reactions to Evaluation Stress Test, CREST; de Weerth, Zijlmans, Mack, & Beijers, 2013a) were used. Cortisol stress responses were determined using six cortisol saliva samples. From these samples two cortisol stress response indices were calculated: total stress cortisol and cortisol stress reactivity. To determine children's cortisol circadian rhythm data on eight cortisol circadian samples collected during two days were used. Total diurnal cortisol and diurnal cortisol decline scores were calculated as indices of the cortisol circadian rhythm. The results showed that higher total diurnal cortisol concentrations and a smaller diurnal cortisol decline were both uniquely associated with higher total stress cortisol concentrations. These associations between the circadian indices and total stress cortisol support that both patterns of cortisol production (circadian and stress response cortisol production) may derive from the same underlying mechanism: HPA-axis functioning. However, there was no evidence for an association between the circadian markers and cortisol stress reactivity in response to the social evaluative stress test. The finding that more total diurnal cortisol and less diurnal decline were associated with more total stress cortisol may further be explained by children's self-regulatory capacities and differences in parenting quality.

Chapter 3

The first goal of this chapter was *to examine the longitudinal development of children's cortisol circadian rhythm from age 1 to 6 (Aim 1.2).* The second goal was *to examine the associations between maternal prenatal and early postnatal distress and (the longitudinal development of) children's cortisol circadian rhythm from age 1 to 6 (Aim 3.1).* To examine this, data of 193 healthy mother-child dyads were used. Maternal distress was assessed prenatally in week 37 of gestation and postnatally at child age 3, 6, 12, 30, and 72 months. Child saliva cortisol samples were collected to determine the cortisol circadian rhythm at 12, 30, and 72 months. Total diurnal and diurnal cortisol decline were calculated for each of these three time point as indices of the cortisol circadian rhythm.

The results indicated that the cortisol circadian rhythm showed developmental changes between the ages of 1 and 6 years. Specifically, total diurnal cortisol concentrations declined between the ages of 1 and 6 and their day-to-day stability increased between the ages of 30 and 72 months. There was no evidence for developmental change in diurnal cortisol decline or its day-to-day stability. Higher levels of maternal pregnancy-specific stress were associated with higher diurnal cortisol concentrations of the children. Higher levels of postnatal maternal anxiety during the first 6 postnatal months were associated with smaller diurnal cortisol declines and higher levels of postnatal maternal stress during the first 6 postnatal months were associated with larger diurnal cortisol declines in the children from age 1 to 6. No support for interaction effects of maternal distress and time were found. The developmental patterns found suggest that the cortisol circadian rhythm shows developmental changes between the age of 1 and 6 years. The associations found between maternal distress and child cortisol markers of HPA-axis functioning may be pointing at a programming effect of maternal distress early in the child's life on the cortisol circadian rhythm of the children between the ages of 1 and 6 years.

Chapter 4

The first goal of this chapter was *to examine the associations between maternal prenatal and early postnatal distress and 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 3.2).* The second goal was *to examine the association between 6-year-old children's cortisol stress responses and gazing during an acute stressor (Aim 2.1).* To examine this, data of 149 6-year-olds who participated in a social evaluative stress test in front of a judge were used. Cortisol stress responses were operationalized by two cortisol stress response indices based on the six cortisol saliva samples that were collected: total stress cortisol and cortisol stress reactivity. Gazing behavior was operationalized by observations of gazing behavior at the judge during the stress test. Maternal prenatal and early postnatal distress were also measured. The results showed that less maternal fear of giving birth, higher prenatal maternal evening cortisol concentrations, and more maternal feelings of anxiety in the first 6 postnatal months of the child's life, were all uniquely associated with higher total stress cortisol concentrations of the children at age 6. In addition, cortisol stress reactivity and gazing behavior during an acute stressful situation were associated. There was no evidence for an association between total stress cortisol and gazing during the stressor nor for a predictive role of maternal distress on child gazing behavior or cortisol stress reactivity. The associations between maternal distress and child total stress cortisol may suggest that maternal distress early in the child's life may program children's later HPA-axis functioning.

Chapter 5

The first goal of this chapter was *to examine the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 2.2).* The second goal was *to examine the moderating role of current maternal parenting stress on the associations between 6-year-old children's cortisol stress responses during an acute stressor and their behavioral functioning at school (Aim 3.3).* To examine this, data of 149 6-year-old's who participated in a social evaluative stress test were used. Saliva cortisol samples were collected six times during the stress test to calculate the cortisol stress response. Teachers' assessments of children's internalizing, externalizing, and prosocial behaviors were used to operationalize behavioral functioning. Maternal reports of parenting stress were also used. There was no evidence of an association between cortisol stress responses and behavioral functioning in the classroom nor of a moderating role of current maternal parenting stress on the link between 6-year-olds' cortisol stress responses and their behavioral functioning at school. Various explanations for these findings are possible including a potential independence of cortisol stress responses and behavior at school and study characteristics, such as teacher reports or contextual factors.

In summary, the studies of this dissertation indicated that total diurnal cortisol concentrations showed developmental change between the ages of 1 and 6 years, and that indices of the cortisol circadian rhythm at the age of 6 were associated with total stress cortisol concentrations. Moreover, maternal distress early in children's lives was associated with cortisol indices of their later HPA-axis functioning. Finally, 6-year-old children's gazing in response to a social stressful situation was associated with cortisol stress reactivity. Together, the results of this dissertation indicated that early childhood is an important developmental period for HPA-axis functioning.

Samenvatting

Summary in Dutch

Een essentieel onderdeel van het menselijk stress-systeem is de hypothalamus-hypofyse-bijnier-as (in het Engels de hypothalamic-pituitary-adrenal axis: HPA-axis) met als belangrijkste hormonale eindproduct het hormoon cortisol (bijv., Lupien, McEwen, Gunnar, & Heim, 2009; Nicolson, 2007). Atypische patronen in de afscheiding van cortisol door de HPA-as zijn geassocieerd met de mentale en psychische gezondheid (bijv., Bremmer et al., 2007; Burke, Davis, Otte, & Mohr, 2006; Buske-Kirschbaum et al., 2003; Hankin, Badanes, Abela, & Watamura, 2010; Jessop & Turner-Cobb, 2008; Luby et al., 2003; McEwen, 2008; Phillips, Ginty, & Hughes, 2013; Sephton & Spiegel, 2003). Deze associaties onderstrepen het belang van een goed begrip van het functioneren van de HPA-as, van de dynamiek en ontwikkeling hiervan en van voorspellers en gerelateerde constructen. Dit is vooral van belang met betrekking tot de kindertijd waarin het functioneren van de HPA-as in ontwikkeling is (bijv., Boyce & Ellis, 2005; Custodio et al., 2007; de Weerth & van Geert, 2002; de Weerth, Zijl, & Buitelaar, 2003; Ivars et al., 2015; Saridjan et al., 2010; Shirtcliff et al., 2012; Spangler, 1991; Watamura, Donzella, Kertes, & Gunnar, 2004), beïnvloed kan worden door interventies (bijv., McLaughlin et al., 2015; Slopen, McLaughlin, & Shonkoff, 2014) en gevoelig is voor omgevingsfactoren (bijv., Beijers, Riksen-Walraven, & de Weerth, 2013a; Chaby, 2016; Fernald, Burke, & Gunnar, 2008; Hunter, Minnis, & Wilson, 2011; Loman & Gunnar 2010; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2012; van der Vegt, van der Ende, Kirschbaum, Verhulst, & Tiemeier, 2009). Een goed begrip van het functioneren van de HPA-as in de kindertijd zou bij kunnen dragen aan toekomstige mogelijkheden voor het ontwikkelen van preventie- en interventieprogramma's gericht op jonge kinderen.

Kennis over het functioneren van de HPA-as bij kinderen bevat nog hiaten. Dit zijn onder andere onbeantwoorde vragen over de dynamiek en ontwikkeling van het functioneren van de HPA-as, associaties tussen het functioneren van de HPA-as en gedrag, en de rol van omgevingsstress gedurende het vroege leven van kinderen op het functioneren van de HPA-as, op gedrag en op associaties tussen het functioneren van de HPA-as en gedrag. Om op deze vragen in te gaan is het onderzoek in deze dissertatie gericht op de volgende drie algemene onderzoeksdoelstellingen die onderverdeeld zijn in zeven specifieke onderzoeksdoelen:

1. Inzicht krijgen in de **dynamiek en ontwikkeling** van het functioneren van de HPA-as van kinderen tot/op de leeftijd van 6 jaar (specifieke doelstellingen 1.1 & 1.2).
2. Inzicht krijgen in associaties tussen het functioneren van de HPA-as en **gedrag** bij 6 jaar oude kinderen (specifieke doelstellingen 2.1 & 2.2).
3. Inzicht krijgen in de rol van **omgeving**sstress gedurende het vroege leven van kinderen op gedrag in een acute stressvolle situatie, op (de ontwikkeling van) het

functioneren van de HPA-as en op associaties tussen het functioneren van de HPA-as en gedragsmatig functioneren tot/op de leeftijd van 6 jaar (specifieke doelstellingen 3.1, 3.2 & 3.3).

De onderzoeksvragen werden onderzocht middels data verzameld binnen het BIBO-project (Radboud Universiteit). BIBO is een lopend prospectief longitudinaal onderzoeksproject waarin een cohort van zich typisch ontwikkelende kinderen uit middenklassegezinnen vanaf de zwangerschap gevolgd wordt. In deze dissertatie zijn van deze groep gegevens gebruikt die waren verzameld in de prenatale fase, gedurende de latere fase van de zwangerschap, en postnataal gedurende de eerste 6 levensjaren na de geboorte van het kind. Hierbij zijn gedragsmatige (gedragsobservaties en vragenlijsten), psychologische (vragenlijsten) en biologische (hormonale) onderzoeksgegevens gecombineerd. Hierna wordt een samenvatting gegeven van de vier empirische hoofdstukken in deze dissertatie, gevolgd door een algemene conclusie.

Hoofdstuk 2

In dit hoofdstuk werd in gegaan op het volgende onderzoeksdoel. *Inzicht krijgen in associaties tussen cortisol stressresponses van 6 jaar oude kinderen gedurende een acute stressvolle situatie en hun cortisol dagritme (doelstelling 1.1).* Om dit te onderzoeken zijn gegevens gebruikt van 6 jaar oude kinderen die deelnamen aan een sociaalevaluatieve stresstest (*N* = 149; de Weerth, Zijlmans, Mack, & Beijers, 2013a). Cortisol stressresponses werden bepaald door middel van zes cortisol speekselmonsters. Op basis van deze monsters werden twee cortisol stressresponse indicatoren berekend: de totale hoeveelheid cortisol tijdens de acute stressvolle situatie en cortisol reactiviteit in reactie op de acute stressvolle situatie. Om het cortisol dagritme van de kinderen te bepalen werd gebruik gemaakt van acht cortisol monsters verzameld gedurende twee dagen. Op basis van deze monsters werden als indicatoren van het cortisol dagritme de totale hoeveelheid cortisol gedurende de dag en de afname in cortisol concentraties gedurende de dag bepaald. Uit de resultaten bleek dat een hogere totale hoeveelheid cortisol gedurende de dag en een kleinere afname in cortisol concentraties gedurende de dag beide samengingen met een hogere totale hoeveelheid cortisol tijdens de acute stressvolle situatie. Deze associaties bieden ondersteuning voor het idee dat beide cortisol productiepatronen (cortisol stressresponses en het cortisol dagritme) voort zouden komen uit hetzelfde onderliggende mechanisme: het functioneren van de HPA-as. Er was echter geen bewijs voor een associatie tussen de indicatoren van het cortisol dagritme en cortisol reactiviteit in reactie op de acute stressvolle situatie. De bevindingen dat een hogere totale hoeveelheid cortisol gedurende de dag en een kleinere afname in cortisol concentraties gedu-

rende de dag beide samengingen met een hogere totale hoeveelheid cortisol tijdens de acute stressvolle situatie zouden verder verklaard kunnen worden met behulp van individuele verschillen in ouderschapskwaliteiten en in zelfcontrolecapaciteiten van kinderen.

Hoofdstuk 3

In dit hoofdstuk werd in gegaan op de volgende onderzoeksdoelen. Ten eerste, *inzicht krijgen in de longitudinale ontwikkeling van het cortisol dagritme van kinderen in de periode van 1 tot 6 jaar (doelstelling 1.2).* Ten tweede, *inzicht krijgen in associaties tussen prenatale en vroeg postnatale stress en angst van moeders en (de longitudinale ontwikkeling van) het cortisol dagritme van kinderen in de periode van 1 tot 6 jaar (doelstelling 3.1).* Om dit te onderzoeken zijn gegevens gebruikt van 193 gezonde moeder-kindkoppels. Stress en angst van de moeder werden gemeten in de prenatale fase, in de 37ste week van de zwangerschap, en in de postnatale fase wanneer het kind 3, 6, 12, 30 en 72 maanden oud was. Op de leeftijden van 12, 30 en 72 maanden werden bij de kinderen cortisol speekselmonsters verzameld om een beeld te krijgen van het cortisol dagritme. Op basis van deze monsters werden op elk van deze drie leeftijden de totale hoeveelheid cortisol gedurende de dag en de afname in cortisol concentraties gedurende de dag bepaald als indicatoren van het cortisol dagritme. Uit de resultaten bleek een ontwikkeling in het cortisol dagritme van de kinderen in de periode van 1 tot 6 jaar. Specifiek nam de totale hoeveelheid cortisol gedurende de dag af in de periode van 1 tot 6 jaar en nam de dag-tot-dagstabiliteit van deze indicator toe in de periode van 30 tot 72 maanden. Er werd geen bewijs gevonden voor ontwikkeling van de afname in cortisol concentraties gedurende de dag, of van de dag-tot-dagstabiliteit van deze indicator in de periode van 1 tot 6 jaar. Daarnaast was een hogere mate van zwangerschapsgerelateerde stress bij de moeder geassocieerd met een hogere totale hoeveelheid cortisol van de kinderen gedurende de dag. Meer gevoelens van angst bij de moeder in de eerste 6 postnatale maanden waren geassocieerd met een kleinere afname in cortisol gedurende de dag bij de kinderen. Bovendien waren meer gevoelens van stress bij de moeder gedurende de eerste 6 maanden na de bevalling geassocieerd met een grotere afname van cortisol gedurende de dag bij de kinderen. De gevonden ontwikkelingspatronen suggereren dat het cortisol dagritme van kinderen zich ontwikkelt in de periode van 1 tot 6 jaar. De gevonden associaties tussen stress en angst van de moeder en cortisol indicatoren van het functioneren van de HPA-as van de kinderen suggereren dat stress en angst van de moeder gedurende het vroege leven van het kind een programmerend effect zouden kunnen hebben op het functioneren van de HPA-as van kinderen in de leeftijd van 1 tot 6 jaar oud.

Hoofdstuk 4

In dit hoofdstuk werd in gegaan op de volgende onderzoeksdoelen. Ten eerste, i*nzicht krijgen in associaties tussen prenatale en vroeg postnatale stress en angst van moeders, en cortisol stressresponses en het kijkgedrag van 6 jaar oude kinderen gedurende een acute stressvolle situatie (doelstelling 3.2).* Ten tweede, *inzicht krijgen in associaties tussen cortisol stressresponses en kijkgedrag gedurende een acute stressvolle situatie bij kinderen van 6 jaar (doelstelling 2.1).* Om dit te onderzoeken zijn gegevens gebruikt van 6 jaar oude kinderen (*N* = 149) die deelnamen aan een sociaalevaluatieve stresstest terwijl hun prestaties werden geobserveerd door een beoordelaar. Cortisol stressresponses werden bepaald door middel van zes cortisol speekselmonsters. Op basis van deze monsters werden twee cortisol stressresponse indicatoren berekend: de totale hoeveelheid cortisol tijdens de acute stressvolle situatie en cortisol reactiviteit in reactie op deze acute stressvolle situatie. Kijkgedrag werd geoperationaliseerd door het observeren van het kijken van de kinderen naar de beoordelaar tijdens de acute stressvolle situatie. Ook prenatale en vroege postnatale stress en angst van de moeders werd gemeten. Uit de resultaten bleek dat minder angst van de moeder voor de bevalling, hogere concentraties avondcortisol van de moeder tijdens de zwangerschap en meer gevoelens van angst bij de moeder gedurende de eerste 6 maanden na de bevalling geassocieerd waren met een hogere totale hoeveelheid cortisol tijdens de acute stressvolle situatie bij de 6 jaar oude kinderen. Daarnaast was cortisol reactiviteit in reactie op de acute stressvolle situatie geassocieerd met kijkgedrag tijdens deze situatie. Er was geen bewijs voor een associatie tussen de totale hoeveelheid cortisol van 6 jaar oude kinderen en hun kijkgedrag tijdens de acute stressvolle situatie of voor een voorspellende rol van stress en angst van de moeder tijdens het vroege leven van het kind op kijkgedrag of cortisol reactiviteit van de kinderen tijdens de acute stressvolle situatie. De gevonden associaties tussen stress en angst van de moeder tijdens het vroege leven van het kind en de totale hoeveelheid cortisol van de kinderen tijdens de acute stressvolle situatie op de leeftijd van 6 jaar suggereren dat stress en angst bij de moeder gedurende het vroege leven van het kind een programmerend effect zouden kunnen hebben op het latere functioneren van de HPA-as van kinderen.

Hoofdstuk 5

In dit hoofdstuk werd in gegaan op de volgende onderzoeksdoelen. Ten eerste, *inzicht krijgen in associaties tussen cortisol stressresponses van 6 jaar oude kinderen gedurende een acute stressvolle situatie en hun gedragsmatig functioneren op school (doelstelling 2.2).* Ten tweede, *inzicht krijgen in de modererende rol van huidige ouderlijke stress van de moeder op de associaties tussen cortisol stressresponses van 6 jaar oude kinderen*

gedurende een acute stressvolle situatie en hun gedragsmatig functioneren op school (doelstelling 3.3). Om dit te onderzoeken zijn gegevens gebruikt van 6 jaar oude kinderen die deelnamen aan een sociaalevaluatieve stresstest (*N* = 149). Gedurende de stresstest werden zes speekselmonsters afgenomen om cortisol stressresponses te meten. Door de leerkracht ingevulde vragenlijsten met betrekking tot internaliserend, externaliserend en prosociaal gedrag van de kinderen werden gebruikt als maten van gedragsmatig functioneren. Ook werden door de moeder ingevulde vragenlijsten met betrekking tot ouderlijke stress gebruikt. Er werd geen bewijs gevonden voor associaties tussen de cortisol stressresponses en gedragsmatig functioneren van de kinderen of voor een modererende rol van ouderlijke stress van de moeder op de mogelijke associaties. Er zijn verschillende verklaringen mogelijk voor deze bevindingen zoals een mogelijke onafhankelijkheid van cortisol stressresponses en gedragsmatig functioneren op school of studiekenmerken zoals het gebruik van vragenlijsten voor gedragsmatig functioneren ingevuld door de leerkracht of contextuele factoren.

Samenvattend lieten de studies in deze dissertatie zien dat de totale hoeveelheid cortisol gedurende de dag een ontwikkeling doormaakt in de periode van 1 tot 6 jaar en dat indicatoren van het cortisol dagritme geassocieerd waren met de totale hoeveelheid cortisol gedurende een acute stressvolle situatie bij kinderen van 6 jaar. Daarnaast lieten de studies zien dat stress en angst van de moeder gedurende het vroege leven van het kind geassocieerd waren met cortisol indicatoren van het functioneren van de HPA-as van de kinderen. Tot slot bleek uit de resultaten een associatie tussen cortisol reactiviteit en kijkgedrag van 6 jaar oude kinderen gedurende een acute stressvolle situatie. Samen suggereren deze bevindingen dat de periode van 1 tot 6 jaar een belangrijke ontwikkelingsfase is voor het functioneren van de HPA-as.

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Curriculum Vitae

Sterre Simons was born on January 1st, 1987, in Arnhem, the Netherlands. After completing her secondary education (Atheneum, nature and health track) at the Arentheem College in Arnhem she studied Psychology at the Radboud University in Nijmegen and obtained her bachelor degree cum laude. Subsequently, she completed the Research Master Behavioural Science (cum laude), which included an internship in the research group of Social Psychology. In September 2012, she started her PhD project at the research group of Developmental Psychology of the Behavioural Science Institute at the Radboud University. In this project, Sterre studied HPA-axis functioning in children using a psychobiological perspective. Currently, she holds a teaching and research position at the research group of Work and Organizational Psychology at the Radboud University.

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